Universidade de Lisboa

Faculdade de Medicina de Lisboa

REVISITING GERM CELL DYNAMICS AND OVARIAN FOLLICLE ASSEMBLY IN THE MOUSE MODEL

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Orientadores: Prof. Doutor Carlos E. Plancha

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Tese especialmente elaborada para obtenção do grau de Doutor em Ciência Biomédicas, especialidade de Ciências Morfológicas

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PREFACE

This thesis investigates the mouse ovarian follicle reserve establishment, focusing on the mechanisms involved in the perinatal extensive germ cell loss and what leads to the retention of a subset of germ cells forming the primordial follicle reserve. Using imaging techniques and animal models we studied the cytoskeleton, extracellular matrix and junctions involvement in this event. The chapters of this dissertation include two published journal articles, (Rodrigues *et al*., 2008 J. Cell. Physiol. 216:355-365; Rodrigues *et al*., 2009 Reproduction 137:709-720).

The present dissertation is outlined as followed:

Chapter 1 – General Introduction divided in two major sections:

1. Oogenesis and folliculogenesis: the beginning, here the developmental trajectory of germ cells until primordial follicle assembly is described.

2. Oogenesis: prospects and challenges for the future. [Rodrigues, P.; Limback, D.; McGinnis, L. K.; Plancha, C. E.; Albertini, D. F. (2008) *J. Cell. Physiol.* 216: 355-365]. This review describes follicle growth and the factors involved in its growth.

Chapter 2 - Multiple mechanisms of gem cell loss in the perinatal mouse ovary. [Rodrigues, P. *et al*., (2009) *Reproduction* 137: 709-720]. This chapter describes multiple mechanisms involved in germ cell loss, around birth, in the mouse model.

Chapter 3 - Perinatal ovarian remodeling and establishment of the definitive follicle reserve: the somatic-germ cell interface. [Rodrigues, P.; Limback, D.; McGinnis, L. K.; Albertini, D. F.; Plancha, C. E.]. Here is

described how germ-somatic-extracellular matrix interactions contribute to ovarian follicle establishment in the mouse.

Chapter 4 - Oocyte and hormonal regulation of the ovarian follicle reserve in the mouse. [Rodrigues, P.; Limback, D.; McGinnis, L. K.; Albertini, D. F.; Plancha, C. E.]. In this chapter both germ cell and hormonal involvement in the ovarian remodeling and ovarian follicle reserve establishment are addressed, using specific genetically manipulated mouse models.

Chapter 5 – Concluding remarks. Here the results are consolidated and further discussed reinforcing the importance of germ-somatic cell communication in the establishment of definitive ovarian follicular reserve in the mouse.

ABBREVIATIONS

ActA	Activin A
ADAMTS-1	A disintegrin and metalloproteinase with thrombospondin-like motives-1
AMH	Anti-Müllerian hormone
AVE	Anterior visceral endoderm
bFGF	Basic fibroblast growth factor
Bcl-2	B-cell lymphoma/leukemia-2
Bax	Bcl-2 associated X protein
BBC3	BCL2-binding component 3
BMP	Bone morphogenetic protein
BMP4	Bone morphogenetic protein 4
BMP7	Bone morphogenetic protein 7
BMP8b	Bone morphogenetic protein 8b
BMP-15	Bone morphogenetic protein-15
Casp4	Caspase 4 (also known as Casp11)
Cx	Connexin
Cx37	Connexin 37
Cx43	Connexin 43
CL	Corpora lutea
CXCR4	CXC chemokine receptor 4
cAMP	Cyclic adenosine monophosphate
COX-2	Cyclooxygenase-2
dpc	Days post-coitum
Dax	Dosage-sensitive sex reversal (DSS)-Adrenal hypoplasia congenital critical region on the X chromosome, gene 1
Dhh	Desert hedgehog
DVE	Dorsal visceral endoderm
E_2	Estradiol-17β
eEF2K	Elongation factor 2 kinase
ERα or β	Estrogen receptor α or β
ECM	Extra cellular matrix
ExE	Extra-embryonic ectoderm
Figα	Factor in the germline-alpha
FGFs	Fibroblast growth factors
FGF9	Fibroblast growth factor 9
FSH	Follicle-stimulating hormone

FSHr	Follicle-stimulating hormone receptor
Foxl2	Forkhead transcription factor 2
Foxo3	Forkhead transcription factor O3
GREL	Gonadal Ridge Epithilial-Like
GV	Germinal vesicle
GVBD	Germinal vesicle breakdown
GCNA-1	Germ cell nuclear antigen-1
GDF9	Growth differentiation factor-9
Gja1	Connexin 43
Gja4	Connexin 37
Ihh	Indian hedgehog
ICM	Inner cell mass
KGF	Keratinocyte growth factor
KL	Kit ligand
KO	Knockout
Lhx8	LIM homeobox protein 8
Lhx9	LIM homeobox protein 9
LIF	Leukemia inhibitory factor
LH	Luteinizing hormone
LHr	Luteinizing hormone receptor
MI	Metaphase I
MII	Metaphase II
MT	Microtubules
MIS	Meiosis-inducing substance
Nobox	Newborn ovary homeobox-encoding gene
NSN	Non-surrounded nucleolus
Oct-4	Octamer-binding transcription factor 4 (also known POU5f1)
PDK1	Phosphatidylinositol-dependent kinase 1
PDGF	Platelet-derived growth factor
PGCs	Primordial germ cells
PG	Prostaglandin
Pin1	Peptidyl-prolyl isomerase 1
PKA	Protein kinase A
POI	Primary Ovarian Insufficiency
PR	Progesterone receptor
PTEN	Phosphatase and tensin homolog deleted on chromosome – 10
PTGS	Prostaglandin endonuclease synthase

RA	Retinoic acid
Sry	Sex-determining gene on the Y chromosome
SSEA-1	Stage specific embryonic antigen-1
Sf1	Steroidogenic factor-1
SDF1	Stromal cell-derived factor
SN	Surrounded nucleolus
Scp3	Synaptonemal complex protein 3
Sl	Still
TNAP	Tissue non-specific alkaline phosphatase
TGFβ	Transforming Growth Factors-β
TZP	Transzonal projections
Act-TZP	Transzonal projections with Actin-filaments
MT-TZP	Transzonal projections with Microtubules
TNFα	Tumour necrosis factor-α
TNFR2	TNF receptor type 2
VE	Visceral endoderm
W	Dominant white spotting
Wt1	Wilms tumour 1
Wnt4	Wingless-related MMTV integration site 4
ZP	Zona pellucida
ZP1	Zona pellucida 1
ZP2	Zona pellucida 2
ZP3	Zona pellucida 3

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INDEX

ABSTRACT

Development of the mammalian ovary is characterized by extensive germ cell loss and the retention of a subset of germ cells within the primordial follicle reserve. Here we aimed to identify the mechanisms whereby some germ cells are retained while others are lost using novel imaging techniques and animal models.

We observed that multiple mechanisms contributed to germ cell loss at birth. Our results confirmed germ cell loss around birth of approximately 44%, but our apoptotic numbers did not account to all that loss. Besides non-apoptotic germ cell loss through the ovarian surface, the use of serum cultured ovaries, and selective autophagy and apoptosis inhibitors, allowed us to suggest other cell death mechanism besides apoptosis may be involved in follicular reserve establishment around birth.

To investigate germ-somatic cell interactions during ovarian follicle assembly, antibodies against cytoskeleton, extracellular matrix (ECM), and intercellular junctions were used to track expression patterns by immunofluorescence. We could confirm that a true ovarian epithelium is not formed until day 20 after birth. The persistence of gap junctions throughout ovary development implies that ovarian follicle reserve establishment requires an ongoing dialogue between somatic and germ cells.

To better understand this cellular dialogue, we used four mouse models with specific gene deletion: GDF9 (growth differentiation factor 9), Nobox (newborn ovary homeobox gene), Sohlh1 (sperma-and oogenesis basic helix-loop-helix1), and FSHβ (follicle stimulating hormone). We found deficient gap junction communication between germ-somatic cells in the FSHβ and GDF9 models, as well as specific cytoskeletal and ECM modifications in all models. In conclusion both oocyte-intrinsic (GDF9, Nobox, Sohlh1) and ovary extrinsic (FSHβ) factors differentially modulate germ-somatic cell interactions during ovarian development, influencing follicle assembly and ovarian follicle reserve establishment.

Key words: ovarian follicle reserve; germ cells; primordial follicles; germ-somatic cell communication;

CHAPTER # 1

GENERAL INTRODUCTION

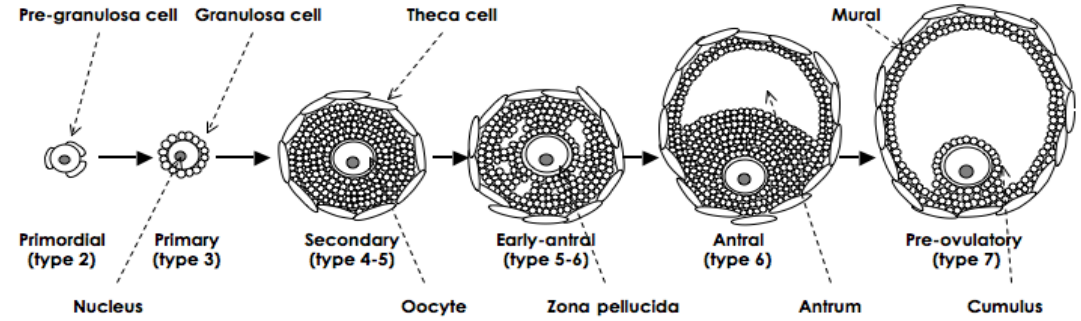
1.1 Oogenesis and folliculogenesis: the beginning

The process of germ cell formation is known as **gametogenesis.** In animal groups that reproduce sexually two different forms of gametogenesis exist: **oogenesis**, differentiation of an ovum (females); and **spermatogenesis**, sperm differentiation (male) (Wassarman and Albertini, 1994; Gilbert, 1997). In both, female and male, germ cells differentiate from **primordial germ cells** (PGCs), thus the first event in gametogenesis is the formation of PGCs. In females, PGCs go through several transformations, as they progress from PGCs to oogonia to oocytes. In mammals, these events normally take place during fetal life, when oogonia enter meiosis and arrest at prophase of meiosis I remaining arrested for months or years depending on the species (Peters and McNatty, 1980; Wassarman and Albertini, 1994). To grow and mature the oocyte must be enclosed by specialized somatic cells: the granulosa cells. The complex of oocyte - granulosa cells forms the initial ovarian follicle (Buccione *et al*., 1990; Eppig, 2001). Follicle growth accompanies oocyte growth and maturation, a process usually designated as **folliculogenesis** (see box 1) (Peters and McNatty, 1980; Eppig, 2001). A theca cell layer differentiates later, in a particularly well-coordinated process, with participation of paracrine and/or autocrine factors (see box 1). The latter stages of follicular growth become dependent on pituitary hormones (Eppig, 2001; Vanderhyden, 2002; Albertini and Barrett, 2003). In the mammalian ovary, the majority of follicles remain dormant and are progressively recruited to the growing pool until the supply of developmentally competent oocytes is exhausted (Hirshfield, 1992). The ultimate goal of oogenesis and folliculogenesis is the production of female sex hormones and healthy oocytes to ensure the development

of healthy babies, ensuring species continuity. How this happens has been the subject of many investigations.

Box 1 Stages of follicle development and oocyte growth

In the mouse follicle assembly occurs shortly after birth, when few flattened pre-granulosa cells associates with the primary oocyte – **primordial follicle** (type 2). Then the flattened cells become cuboidal (granulosa cells) and completely surround the oocyte with a single layer of cells – **primary follicle** (type 3). Multiple layers of granulosa cells surround the oocyte, zona pellucida begins to form, and theca cells start surrounding the follicle – **secondary follicle** (type 4-5). Small patches of fluid become evident within the granulosa cells, which continue to proliferate – **early-antral follicle** (type 5-6). The patches of fluid become a single cavity, the antrum – **antral follicle** (type 6). Continuing to grow, the follicle presents now two types of granulosa cells depending on their location: on the follicular wall – mural; and surrounding the oocyte – cumulus, granulosa cells, and the antrum cavity is now increased – **pre-ovulatory follicle** (type 7). See figure 8 for ovarian mouse examples.



References: Pedersen and Peters (1968); Peters and McNatty 1980)

Origin of germ cells: historical perspective

Primordial germ cells are the precursors of oocytes and sperm. The origin of PGCs is the matter of many discussions and controversies, before becoming a scientific dogma. More than a century ago, Weismann (Heys, 1931) published his theory on "clear distinction between the soma and germ plasm". This scientist developed his studies in Hydromedusae, proposing the existence of a "germ plasm" (preformed germ cell determinates), which were transmitted only to the future germ cells to ensure totipotency and germline continuity (Saitou *et al*., 2003). In some organisms including *Caenorhabditis elegans*, *Drosophila melanogaster* and *Xenopus laevis*, this has been proven to be true, in fact some embryonic cells, in these organisms inherited morphologically and functionally distinct cytoplasmic components which will later develop as germ cells (Wylie, 1999; Extavour and Akam, 2003). Previous to Weismann, Waldeyer firstly

addressed the idea of the germ cells uniqueness and early separation from somatic cells in vertebrates (Heys, 1931; Everett, 1945). This author observed germ cells within the "germinal epithelium" of the chicken ovary; furthermore he reported that these germ cells had been produced by the epithelium itself (Heys, 1931; Everett, 1945). Since then many theories were formed and summarized (Heys, 1931; Everett, 1945). An interesting and controversial question derived: "Is the number of germ cells finite or is there *de novo* formation of germ cells whenever those are needed?" This question remains open even today.

Regarding germ cell origin, it was not until the 1950's when Chiquoine (Chiquoine, 1954) used tissue non-specific alkaline phosphatase (TNAP) as a marker for germ cells that early germ cell segregation was accepted without doubt (Ginsburg *et al*., 1990). This indicated that, unlike flies and frogs, mammals do not have preformed germ cell determinants (Wylie, 1999; Extavour and Akam, 2003). Hence, mammalian germ cell formation involves epigenesis (progressive development from an undifferentiated cell) and not preformation (Pinto-Correia, 1997; Watson and Tam, 2001; Extavour and Akam, 2003; Saitou *et al*., 2003).

In the mouse, PGCs originate in the base of the developing allontois, between endoderm and mesoderm of the ventral amniotic fold, at 7-7.5 days post-coitum (dpc) (Ginsburg *et al*., 1990; McLaren, 2003). In humans, these cells first appear around 3 weeks post-fertilization (Motta *et al*., 2003). After their segregation from somatic cell lineages, PGCs migrate through the embryo to become incorporated in the developing gonads where they finalize their differentiation. During migration PGCs actively proliferate. (Buehr, 1997; McLaren, 2003)

Primordial Germ Cells specification

Lawson and Hage (1994) were able to locate the PGC ancestral population in the most proximal epiblast cells at 6-6.5 dpc (early gastrulation), by injecting single epiblast cells with a lineage marker of

mouse embryos. PGC's were not yet fully differentiated since some labelled cells contributed to other somatic cell-lineages (Lawson and Hage, 1994). However, this work determined that the founding population of precursor germ cells were allocated early during gastrulation (7.2 dpc) as a group of approximately 40 to 45 cells (Ginsburg *et al*., 1990; Lawson and Hage, 1994). Studies point to mammalian germ cell-lineages being dependent on external signals rather than being predetermined (Extavour and Akam, 2003; McLaren, 2003; De Felici *et al*., 2004). Contributing to this conclusion were observations on mutant Bone Morphogenic Protein-4 (*Bmp4*) mice, which lack PGCs (Lawson *et al*., 1999) (Fig.1). This observation suggested the involvement of BMP4, an intracellular signalling protein member of the Transforming Growth Factors-β (TGFβ) superfamily, in the specification of germ cells. However, BMP4 signalling was not restricted only to germ cells since these animals also lack allantois (Lawson *et al*., 1999). *Bmp8b* mutants also showed a severe, although not as drastic reduction of PGC numbers (Ying *et al*., 2000). In vitro studies showed that both BMP4 and BMP8b signalling in the epiblast cells have a synergistic action on germline competency acquisition. However, BMP4 is required for epiblast cells to gain germ cell competency before (Ying *et al*., 2001) (Fig.1). Further studies found that BMP4 induces *fragilis* expression on the epiblast tissue (Saitou *et al*., 2002). *Fragilis* is a putative interferon-inducible gene, which expression in this epiblast cells is thought to induce a transmembrane protein associated with germ cell acquisition of competence (Saitou *et al*., 2002; Lange *et al*., 2003). In fact, it has been shown that only the cells with highest expression of *fragilis* will express *stella*, the first gene known to be germ cell lineage-restricted (Saitou *et al*., 2002). *Stella* is a nuclear-cytoplasmic protein that seems to be involved in chromosomal and RNA organization and is predominantly expressed in totipotent and pluripotent cells (Saitou *et al*., 2003) (Fig.1). Importantly, *stella*-positive cells exhibited repressed homeobox genes, suggesting that founder cells acquire the capacity to avoid somatic cell fate (due to

homeobox genes repression) and maintenance of pluripontency (Saitou *et al*., 2002). The repression of homeobox genes seems to be due to the expression of *Blimp1* (*Prdm1*), a potent transcriptional repressor of a histone methyltransferase subfamily, in the epiblast at 6.25 dpc and later restricted to the founder germ cell cluster (Ohinata *et al*., 2005; Saitou *et al*., 2005) (Fig.1). Another cell pluripontency associated gene is *Oct-4*, a POU transcription factor encoded by the *Pou5f1* gene, which was demonstrated to be expressed in PGCs, unfertilized oocytes and in the inner cell mass (ICM) of blastocysts (Scholer *et al*., 1990b; Watson and Tam, 2001). Like alkaline phosphatase activity, at 7.0 dpc Oct 4 is still expressed in the epiblast cell but becomes germ cell restricted at 7.5-8.0dpc (Scholer *et al*., 1990a) (Fig.1) and is essential for PGCs survival, since Oct-4 loss leads to germ cell apoptosis (Kehler *et al*., 2004). These observations indicate that all cells in the proximal epiblast can be in the founder cluster of PGCs, as long as they are in the right place at the right time for specification (Watson and Tam, 2001).

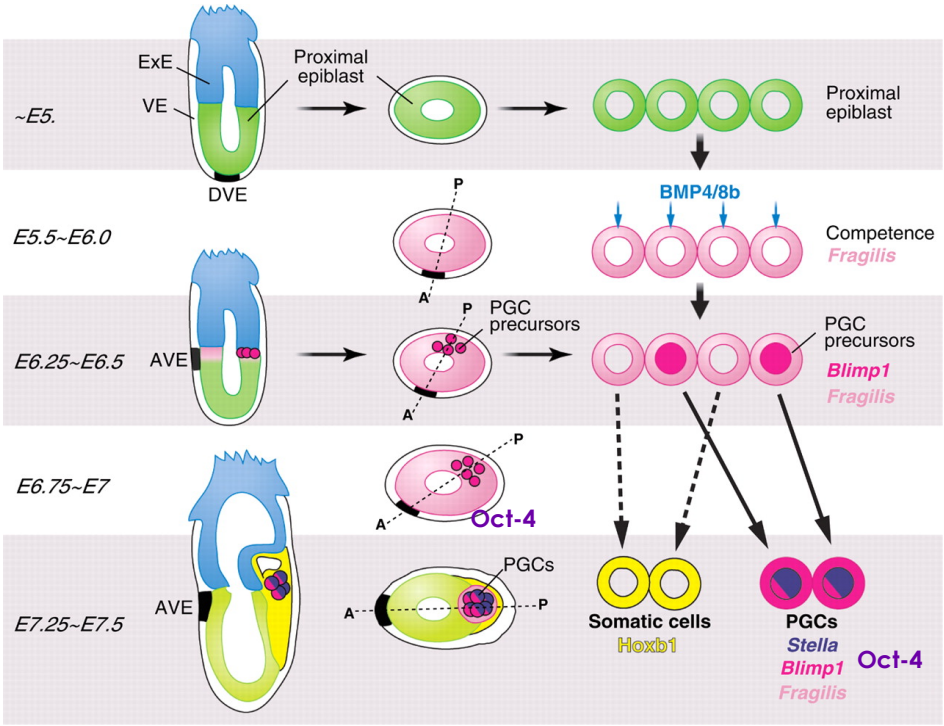


Figure 1 – Primordial Germ Cell specification in the mouse adapted from (Hayashi *et al*., 2007). (VE-visceral endoderm; ExE-extra-embryonic ectoderm; DVE-dorsal visceral endoderm; AVE-anterior visceral endoderm)

Primordial Germ Cell migration and proliferation: arrival to the genital ridges

At this point in time (7.0-7.5dpc) in the mouse embryo PGC cluster is outside of the embryo proper at the base of the allantois. A day later, 8.5dpc, PGCs are associated with the endodermic cells. As the endodermic cells begin invagination to form the hindgut, PGCs begin migration (Fig. 2) (Byskov, 1982; Motta *et al*., 1997; McLaren, 2003; Motta *et al*., 2003). Approximately, one day later (9.5dpc) in the mouse PGCs translocate from the hindgut and actively move dorsally into the mesentery (Fig. 2) (Molyneaux *et al*., 2001). Next, at 10.5dpc PGCs start migrating directionally from the dorsal body wall to the genital ridges - the future gonads (Fig. 2). Finally at 11.5dpc mouse PGCs populate genital ridges (Fig. 2). (Byskov, 1982; Molyneaux *et al*., 2001; McLaren, 2003; Molyneaux and Wylie, 2004) During migration PGCs actively proliferate, continuing for 2-3 days after arrival at the genital ridges, allowing the initial 45 cells to become approximately 20 000 at 13.5dpc (Tam and Snow, 1981).

Several mechanisms may be used by the PGCs and surrounding cells for migration: adhesion, where PGCs respond to cell-cell contacts; contact guidance, PGCs move along a preformed molecular pathway in a substrate; and/or chemotaxis, PGCs are attracted by a substance produced elsewhere (Buehr, 1997). It was proposed that PGCs arrive to the hindgut independently but when leaving the gut to the dorsal mesentery (9.5dpc) they form aggregates, extending long process of stage specific embryonic antigen-1 (SSEA-1) forming a network (Gomperts *et al*., 1994). SSEA-1 has been proposed as a specific cell-surface marker of undifferentiated cells and is often used to identify pluripotent cells (Choi *et al*., 1999). More recently it was shown that E-cadherin is essential to the PGC-PGC connection and motility in the migratory process. Disrupting PGC-PGC adhesion through E-cadherin functional antibodies induced the formation of ectopic germ cells (Bendel-Stenzel *et al*., 2000; Di Carlo and De Felici, 2000). Interactions between PGCs and the extra cellular matrix

(ECM) in which PGCs translocate, and β1-integrins, cell surface receptors in the ECM, were also shown to be important for PGCs locomotion during migration (Garcia-Castro *et al.*,1997; Anderson *et al.*,1999).

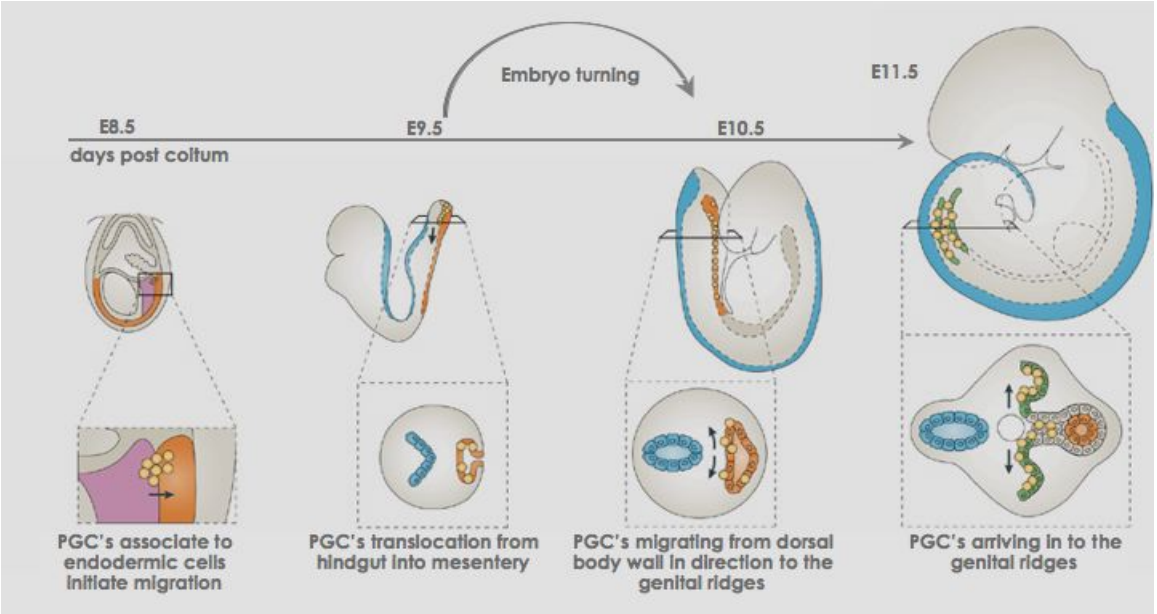


Figure 2 - Primordial germ cells migration into the genital ridges. Adapted from (Richardson and Lehmann, 2010).

Disruptions in the c-kit/kit-ligand (KL/stem cell factor/mast cell growth factor) leads to germ cell migration problems in addition to alterations in patterns of survival and proliferation (Keshet *et al.*, 1991; Buehr *et al.*, 1993; Pesce *et al.*, 1997). The tyrosine kinase receptor, c-kit and its ligand-KL are important regulators of germ cell development, encoded in the genes White and Steel, respectively and are among the loci most studied in the mouse (Russel *et al.*, 1968; Keshet *et al.*, 1991). *In vitro* studies lead to the understanding of the importance of these proteins and the surrounding environment in migration, proliferation and survival (Godin *et al.*, 1991; Pesce *et al.*, 1997). Recently, Steel factor was proven to be essential for PGCs migration and proliferation (Runyan *et al.*, 2006). Other important factors on germ cells proliferation are the fibroblast growth factors (FGFs) associated with proliferation of many cell types, including germ cells and mesoderm formation in embryos (Kawase *et al.*, 2004). FGF-4/8/17 are expressed in the neighbouring cells of PGCs, during migration but FGF-4/8 is

expressed by the PGCs when they arrive to the genital ridges. This differential expression of FGF may indicate that during migration proliferation is induced through a paracrine mechanism while within the genital ridges it may switch to an autocrine mechanism (Kawase *et al*., 2004). These authors also found that without c-kit, FGFs together with KL promote proliferation but the presences of c-kit induced an inhibitory effect on FGF's-stimulated PGCs proliferation. Peptidyl-prolyl isomerase 1 (Pin1) involvement in PGCs proliferation was demonstrated through $Pin1^{-/-}$ females, which are born with significantly less oocytes, although PGCs allocation was perfectly normal (Atchison *et al*., 2003). Further studies showed that PGCs expansion was impaired with no signs of cell cycle arrest or apoptosis, thus indicating prolonged cell cycles, fewer divisions, which in the end results in lower oocyte numbers (Atchison *et al*., 2003).

Studies in the early nineties suggested that chemotropic substances released by the genital ridges, the target tissue, act as attractants to germ cells - chemotaxis (Godin *et al*., 1991; Godin and Wylie, 1991; Buehr, 1997). An *in vitro* study showed that soluble TGFβ1 in the culture media mimicked the signals produced by the genital ridges at 10.5dpc (Godin and Wylie, 1991). More recently it was shown that colonization of genital ridges requires the ligand-receptor interaction – SDF1/CXCR4 (Molyneaux *et al*., 2003). Stromal cell-derived factor – SDF1 is a member of chemokine CXC subfamily and the only known ligand for the G-protein-coupled receptor CXC chemokine receptor 4 - CXCR4, also a co-receptor for HIV-1 virus in humans (Bleul *et al*., 1996; Molyneaux *et al*., 2003; Peng *et al*., 2004). The ligand, SDF1, is expressed in the dorsal body wall during migration while the receptor, CXCR4, is expressed in the migrating germ cells (Molyneaux *et al*., 2003). These authors showed that exogenous SDF1 induced abnormal migration, and targeted mutations to CXCR4 lead to genital ridge colonization problems (Molyneaux *et al*., 2003). Similarly, $SDF1^{-/-}$ showed impaired colonization of the genital ridges, indicating that SDF1 is not required for migration but for homing the PGCs (Ara *et al*., 2003).

The proliferation within the genital ridges is a special program in which PGCs expand in number but also form cysts or clusters, which divide synchronously but without cytokinesis. Thus PGCs develop in a cluster of 4 to 32 cells with cytoplasm connected by an intercellular bridge (Pepling and Spradling, 1998; Pepling *et al*., 1999). This is a conversed mechanism from invertebrates to vertebrates that occurs before germ cells enter meiosis at the time of sex determination (Pepling *et al*., 1999). This conservation demonstrates the importance of the intercellular bridge connections between germ cells before meiosis. This mechanism is very well studied in *Drosophila*, which grow up to 16 germ cells before entering meiosis (de Cuevas and Spradling, 1998). However, striking similarities have been identify in *Xenoupus*, in which germ cells also divide synchronously to form a cluster of 16 cells entering meiosis afterwards (Pepling *et al*., 1999) and in the mouse it is predicted for the clusters to grow to 32 germ cells before entering in meiosis (Pepling and Spradling, 1998).

Sex determination

When germ cells reached the genital ridges, they are still undifferentiated. Sex determinant mechanisms vary among the animal kingdom: ratio of X chromosomes to autosomes (worms and flies); or temperature, hormones, environmental cues (reptiles and fishes) (Tilmann and Capel, 2002). In mammals, until the early 2000's, the ovary was believed to be a default pathway, set to always develop in the absence of "male/testis signals" (Lovell-Badge and Hacker, 1995; Tilmann and Capel, 2002). However, several genes have been implicated in gonad formation in both sexes. Among these are steroidogenic factor-1 (*Sf1*), Wilms tumour 1 (*Wt1*), and LIM homeobox protein 9 (*Lhx9*) (McLaren, 2000; Tilmann and Capel, 2002), which have been implicated by the absence and/or defects in genital ridges of knockout (KO) mouse models (Pangas and Rajkovic, 2006).

In males, the decision to make testes is controlled by the sex-determining gene on the Y chromosome – *Sry* (Koopman *et al*., 1991). This gene is

expressed in the mouse embryo from approximately 10.5 to 12.5dpc, only in the supporting cell lineage within the genital ridge (Lovell-Badge and Hacker, 1995; Koopman, 2001; McLaren, 2003). *Sry* induces the differentiation of Sertoli precursors and its down-regulation coincides with the beginning of testes development (Tilmann and Capel, 2002; Kim and Capel, 2006). FGF9 is involved in the migration of mesonephric cells (Colvin *et al*., 2001) and in germ cell survival, hence it is one of the first factors on the sex-determinant pathway (DiNapoli *et al*., 2006). This growth factor also "antagonizes" with wingless-related MMTV integration site 4 (Wnt4) and together they form a balanced mechanism of decision between male and female, respectively (Kim *et al*., 2006).

In female mice, deletion of Wnt4 leads to their masculinization, due to testosterone production, suggesting Wnt4 as a determinant gene for female sex differentiation (Vainio *et al*., 1999; Jordan *et al*., 2001; Yao *et al*., 2004; Heikkila *et al*., 2005; Kim and Capel, 2006). Wnt4 is expressed in the undifferentiated gonads of mice until 11.5dpc, when it is down regulated in the males but persists in the females (Vainio *et al*., 1999). Wnt4 controls the expression of follistatin, which is responsible for repressing the formation coelemic vessel (testis formation precursor) and contributes to germ cell survival in the ovary (Yao *et al*., 2004). Along with Wnt4, Dax1 (Dosage-sensitive sex reversal (DSS)-Adrenal hypoplasia congenital critical region on the X chromosome, gene 1) was also thought to be an ovary regulatory gene (Zanaria *et al*., 1995). In fact, Dax1, like Wnt4, is down regulated in males when differentiation begins (~12.5dpc) while persisting in the ovary (Swain *et al*., 1996). Since its overexpression lead to XY sex reversal, antagonizing with Sry (Swain *et al*., 1998) it was the a perfect candidate for an ovary determinant gene. However, it is not required for ovary development but for testis development instead, working more like an "anti-testis than an ovary determinant" (Yu *et al*., 1998). Furthermore, Wnt4 and Dax1 have a joint role in controlling female development and testes repression, where Wnt4 up regulates Dax1 (Jordan *et al*., 2001; Mizusaki *et*

al., 2003). The finding that female mice lacking the gene forkhead transcription factor 2 (*Foxl2*) have oocytes but also have differentiated male somatic cells, lead to the suggestion that sex determination continues throughout ovarian development (Loffler *et al*., 2003; Baron *et al*., 2005; Ottolenghi *et al*., 2005). Foxl2 expression initiates when gonads start differentiating (~12.5dpc) (Loffler *et al*., 2003), which could implicate it as being a female determinant gene, however the fact that it occurs after oocyte loss, may also be indicative of an oocyte effect (Ottolenghi *et al*., 2005). Hence Wnt4 continues to be the primary female gene determinant studied so far (Fig. 3).

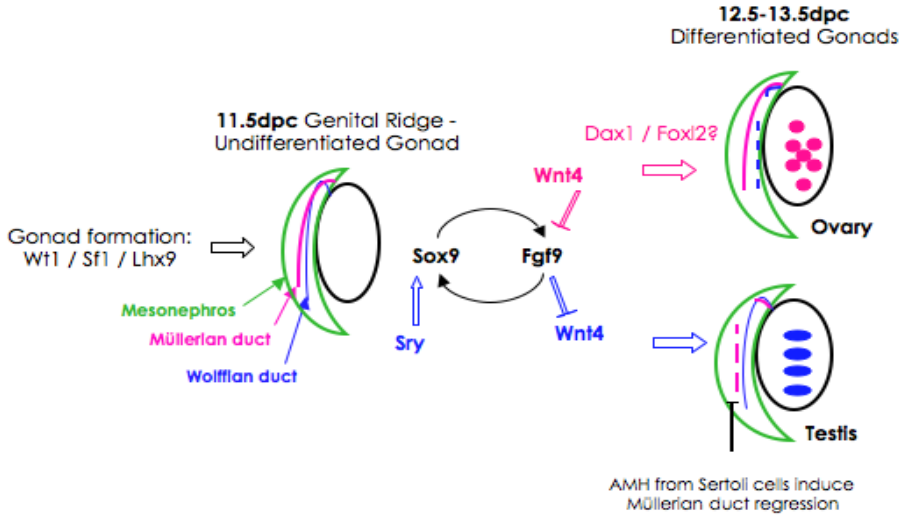


Figure 3. Schematic summary of gonad development and sex determination in mice. Initially to form a bipotential gonad Wt1, Sf1 and Lhx9 are needed. On day 11.5 dpc primordial germ cells arrive into the genital ridge, with no phenotypical difference between sexes. If Sry is present Sox9 is up regulated and in a forward loop induced Fgf9, which inhibits Wnt4, and testis is formed. Müllerian ducts start regression due to Sertoli cells AMH production. However if Wnt4 is dominant, Fgf9 is inhibited and consequently Sox9; Dax1is is up regulated and Foxl2 begins expressing, an ovary is formed. In this case it is the Wolffian duct that regresses.

Germ cell meiosis initiation

Twelve and half days post-coitum, PGCs within the genital ridges initiated differentiation and continue proliferating until approximately 13.5dpc. Around this time, occurs the first germ sex developmental difference between male and female germ cells (Swain, 2006). In the males, germ cells (T-prospermatogonia) arrest in the G1/G0 stage of the mitotic cell

cycle around 13dpc (McLaren and Southee 1997). On the other hand, female germ cells (oogonia) enter prophase of the first meiotic division, arresting in pachytene stage prior to birth, resuming meiosis shortly before ovulation (McLaren and Southee, 1997).

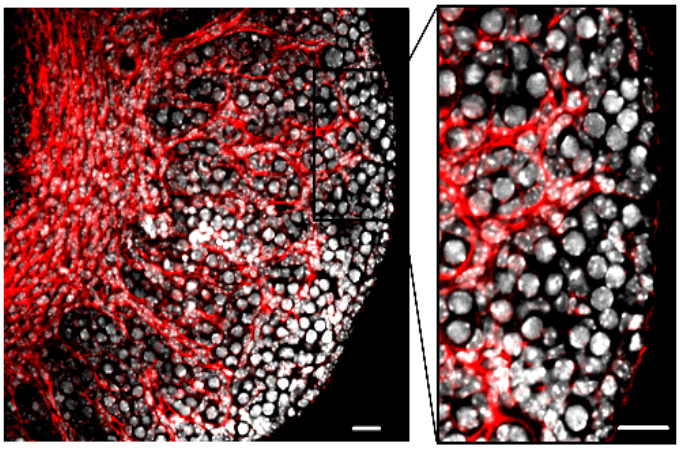


Figure 4. Mouse fetal ovary, 16 days post *coitum*, cluster delimitation by actin-filaments in red and nuclei in white, detailed image of actin-clustered oogonia on the right. Scale bars 20µm.

Around day 13.5 post-coitum in the mouse, oogonia are interconnected with one another by inter-cytoplasmic bridges and surrounded by somatic cells forming the ovigerous cords (Byskov and Lintern-Moore, 1973; Pepling *et al*., 1999) (Fig. 4). At the same time Stra-8 is expressed and oogonia transit from the mitotic to the meiotic cell cycle, becoming primary oocytes (Hirshfield, 1991; Koubova *et al*., 2006). They progress through meiotic prophase-I stopping at diplotene. By day 17.5 post-coitum some oocytes are already arrested in diplotene, while others will do so shortly after birth (Hirshfield, 1991).

Germ cell reserve establishment and primordial follicle assembly

In most mammals, female meiosis entry coincides with a major germ cell loss, which in rodents goes up to 80% of the initial oogonia pool (Baker, 1966; Hirshfield, 1991; Tilly, 2001). The reason for the massive loss may be associated with errors in mitosis, or chromosome pairing, (Tilly, 2001; McClellan *et al*., 2003; Di Giacomo *et al*., 2005). The proposed mechanisms usually associated with germ cell elimination are: apoptosis (Baker 1966; Peters and McNatty, 1980; Coucouvanis *et al*., 1993; De Pol *et al*., 1997; Pepling and Spradling, 2001; Tilly, 2001; Baum *et al*., 2005) and germ cell extrusion through the ovarian epithelium – or shedding (Byskov and

Rasmussen, 1973; Hirshfield, 1991; Wassarman and Albertini, 1994; Motta *et al*., 2003). We will address this subject further in chapter 2. With few exceptions, (as the lemurs of Madagascar (Gosden, 1995)), the number of germ cells/oocytes is set at birth or shortly after birth and will gradually decrease when ovulation begins (Peters and McNatty, 1980; Byskov, 1982). Recently, this dogma has been challenged in the mouse by Johnson *et al.*, (2004; 2005), Lee *et al.*, (2007), and in human by Bukovsky *et al.*, (2004), generating a very exciting discussion in the recent years among the reproductive scientific community.

Follicle formation occurs after birth and coincides with cluster breakdown, and major germ cell loss (Pepling and Spradling, 2001). In the embryonic mammalian ovary the oogonia clusters are enclosed within somatic cells structures - the ovigerous cords (Fig 4) (Byskov and Lintern-Moore, 1973). The breakdown of both, ovigerous cords and clusters leads to the formation of primordial follicles; the somatic cells from the cords "invade" the cluster and associate with the surviving primary oocytes (Byskov, 1986; Rajah *et al*., 1992; Pepling and Spradling, 2001; Sawyer *et al*., 2002). These invading cells - pre-granulosa cells, are flattened and do not completely enclose the oocyte, this being what characterizes a primordial follicle (Pedersen and Peters, 1968). The assembly of primordial follicles is still not well understood, and will be discussed in chapter 3. Figure 5 summarizes schematically the possible factors involved in cluster breakdown and primordial follicle assembly.

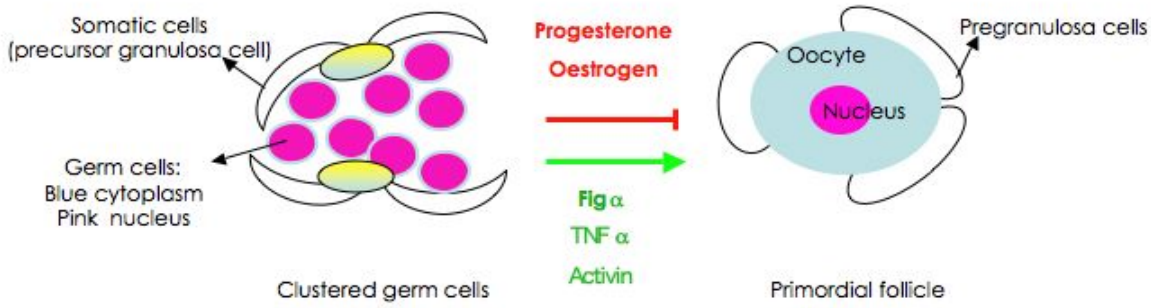


Figure 5. Scheme summarizing primordial follicle assembly, with inhibiting (red - (Chen *et al*., 2007) (Kezele and Skinner, 2003)) and inducing (green - Figα (Soyal *et al*., 2000);

(Marcinkiewicz *et al*., 2002; Morrison and Marcinkiewicz, 2002) (Bristol-Gould and Woodruff, 2006; Coutts *et al.,* 2007)) factors. Yellow/blue cells are the GREL – gonadal ridge epithelial-like cells (Hummitzsch *et al*., 2013).

Primordial follicles remain "quiescent" until initial recruitment into the growing pool (McGee and Hsueh, 2000; van den Hurk and Zhao, 2005). Primordial follicles can arrest at this stage up to 1 year (rodents), or to 5 decades (human), waiting for recruitment (van den Hurk and Zhao, 2005).

Having overviewed primordial germ cells trajectory to follicle assembly, we will now focus on the central player: the oocyte.

1.2. OOGENESIS: PROSPECTS AND CHALLENGES FOR THE FUTURE

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ABSTRACT

Oogenesis serves a singular role in the reproductive success of plants and animals. Of their remarkable differentiation pathway what stands out is the ability of oocytes to transform from a single cell into the totipotent lineages that seed the early embryo. As our understanding that commonalities between diverse organisms at the genetic, cellular and molecular levels are conserved to achieve successful reproduction, the notion that embryogenesis presupposes oogenesis has entered the day-to-day parlance of regenerative medicine and stem cell biology. With emphasis on the mammalian oocyte, this review will cover 1) current concepts regarding the birth, survival and growth of oocytes that depends on complex patterns of cell communication between germ line and soma, 2) the notion of "maternal inheritance" from a genetic and epigenetic perspective, and 3) the relative value of model systems with reference to current clinical and biotechnology applications.

INTRODUCTION

Oogenesis is a protracted process that encompasses the birth, growth, and maturation of a cell unique in its ability to propagate another generation of organisms. In some sense, it is not a very efficient process when measured in terms of viable offspring. In fact, the relative fecundity of a particular organism varies widely according to the kind of reproductive strategy employed. For example, broadcast spawners like fish and many invertebrates are efficient at oogenesis but the fate of ovulated eggs is left up to the whims of the environment the resultant embryos find themselves in. These kinds of animals make a significant metabolic investment in oogenesis. On the other hand, primates expand oocyte numbers prior to birth and engage in a dramatic course of prolonged attrition with a small fraction of the egg endowment surviving to ovulation throughout the reproductive lifespan. As extreme as organisms may be in both the efficiency of egg production and the size of their "spawn" (hundreds to thousands for invertebrates; one for humans), the goals of oogenesis remain the same: producing a developmentally competent egg capable of generating live offspring.

While developmental biologists have long been fascinated by the mechanisms by which an oocyte acquires and executes its totipotentiality during embryogenesis, a new generation of experimentalists have joined the campaign bringing with them research goals of great global and clinical importance. The emergence of the field of Assisted Reproductive Technologies (ARTs) is dominant amongst these new areas of oocyte biology because of the increasing usage of ARTs to treat human infertility. Since the advent of this technology in the late 1970s, more than 3 million children have been born using ARTs and the prospects for innovations and other modifications in current applications are clear. Oocyte and ovary cryopreservation efforts are ongoing as a means to preserve or restore reproductive function to women who have undergone medical treatments that cause partial or complete sterility. Technology for preservation of oocytes, also known as egg banking, is also being viewed as a way to

maintain the diversity of living organisms that are rare, endangered, or bred for agricultural or medicinal value. The key point here is that oogenesis is a gradual process by which the properties needed to express developmental competence are acquired at different stages of differentiation and so the timing for intervention strategies and their impact on oocyte quality represent key challenges for the future. In this sense, virtually all clinical applications aimed at female germ line preservation will require identification of appropriate model systems and to unravel the mechanisms that confer and mediate the expression of oocyte developmental competencies.

With this background, the goals of this review are to 1) highlight current mechanistic viewpoints as related to the stages during which mammalian oocytes are born, grow, and mature, 2) revisit the seasoned concept of "maternal inheritance" in light of new data showing that especially in mammals, the relative contributions of the oocytes' endowment to embryo survival and fetal development have been underestimated, and 3) given the impact that studies of oogenesis will have on biotechnology, stem cell research and animal fecundity, the utility of specific animal models will be discussed in the context of future challenges relevant to human health.

A. Oogenesis is a protracted process that invokes feedback regulation at many levels.

1. Primordial to primary follicle transition

Immediately after primordial follicle assembly some are recruited from the resting pool into the growing population. The mechanism by which primordial follicles are maintained or leave the resting pool is still not well understood. Primordial follicle activation is progressive and initiated with granulosa cell proliferation, and their change in shape from squamous to cuboidal (Hirshfield, 1991; Braw-Tal, 2002). When these cuboidal granulosa cells form a layer that completely surrounds the also enlarging oocyte, the primordial follicle has become a primary follicle (Fig. 6A-C) (Hirshfield, 1991;

Braw-Tal, 2002). Several factors are involved in inhibiting and inducing this transition (Skinner, 2005).

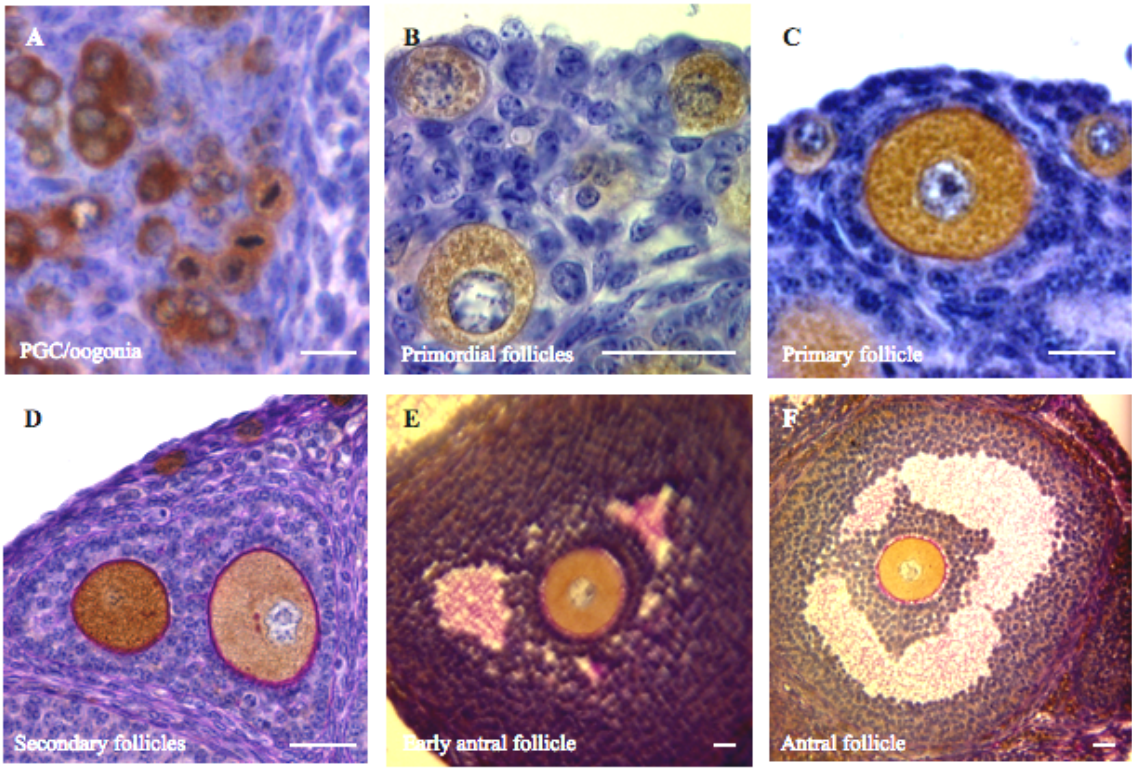

Figure 6. (**A-F**) Examples of stages in follicle and oocyte growth from oogonia (**A**) to antral follicle (**F**). Oocyte cytoplasm is labeled with mouse vasa homologue (MVH) antibody, a specific oocyte factor; zona pellucida is stained with PAS reaction (pink). Note in **B** the onset of zona pellucida formation in the primary follicle. Scale bars 20µm.

Anti-Müllerian hormone (AMH), the growth factor that leads to Müllerian duct regression in males, has been sited as responsible for maintaining primordial follicles in the resting pool in females (Durlinger *et al*., 1999; Durlinger *et al*., 2002). The observation of early age depletion of primordial follicles in AMH-deficient mice and the fact that AMH is produced in the granulosa cells of developing follicles has lead to the suggestion of AMH as an inhibitor of primordial formation (Durlinger *et al*., 1999; Durlinger *et al*., 2002). To further understand the regulatory action of AMH, Nilsson and colleagues (2007) performed a transcriptome analysis of ovaries treated with AMH and known stimulatory factors. Results showed that AMH may decrease the expression of pro-activation factors and increase the

expression of inhibitory factors (Nilsson *et al*., 2007). Forkhead transcription factor O3 (Foxo3; FOXO subfamily of forkhead transcription factors which are all downstream effectors of the PTEN/PI3K/AKT pathway) was also implicated in inhibition of primordial follicle activation (Castrillon *et al*., 2003). These scientists generated mice with a null mutation on the Foxo3 *locus*, and, despite being fairly normal, females exhibited a distinct phenotype of mass follicle activation, leading to early follicular depletion (Castrillon *et al*., 2003). In fact, a detailed characterization of the Foxo3 deficient ovary showed that Foxo3 is necessary to suppress primordial follicle activation (John *et al*., 2007); these animals have viable litters as long as there are follicles available but total follicle depletion happens around 15 weeks of age (John *et al*., 2007). Accordingly, mice where constitutively active Foxo3a was being expressed in the oocytes exhibited infertile females due to slow follicle development and oocyte growth (John *et al*., 2007). Concomitantly, deletion of PTEN (phosphatase and tensin homolog deleted on chromosome 10; upstream of the Foxo3) gene in mouse oocytes result in activation of the majority of primordial follicles by postnatal day 23, and complete loss by 16 weeks post-birth (Reddy *et al*., 2008). PTEN is a lipid phosphatase that negatively regulates the PI3K (phosphatidylinositol 3-kinase) signaling pathway of cell proliferation and survival (Cantley and Neel, 1999). It is involved in regulation of primordial follicle activation, since it is this pathway (PI3K-AKT-Foxo3) that kit ligand [KL, growth factor that binds to its cognate receptor – c-kit (Hutt *et al*., 2006b)] uses to induce oocyte activation (Reddy *et al*., 2005; Liu *et al*., 2006). Another signaling pathway, the chemokine SDF-1 and its receptor CXCR4, has been suggested to negatively regulate primordial follicle activation (Holt *et al*., 2006). Interestingly, *in vitro* addition of recombinant SDF-1 to neonatal ovaries increased the follicle density but decreased the number of growing follicles when compared to controls, indicating a possible role of this pathway in maintaining primordial follicles in the resting pool (Holt *et al*., 2006).

On the other hand, KL produced by granulosa cells, is involved in primordial follicle activation (Parrott and Skinner, 1999). Ovaries cultured with and without recombinant KL and/or neutralizing c-kit antibody (ACK-2) demonstrated that KL is necessary and sufficient to promote primordial follicle recruitment into the growing pool (Parrott and Skinner, 1999). Previously, it had been shown that c-kit (produced by the oocyte) and KL are required in ovarian follicle development prior to gonadotropin dependence (Yoshida *et al*., 1997). Injecting mice with the antibody blocking c-kit function (ACK-2), the authors concluded that KL/c-kit interaction is important for follicle activation in the first 5 days post-birth (Yoshida *et al*., 1997). Interestingly, leukemia inhibitory factor [LIF; an interleukin 6 class cytokine that affects the growth and development of cells (Taupin *et al*., 1998)], is also produced by the granulosa cells as another factor shown to promote primordial to primary follicle transition, increasing KL mRNA production in cultured granulosa cells (Nilsson and Skinner, 2002). LIF may act to induce primordial activation through induction of KL expression, as proved by culture of ovaries in the absence or presence of LIF or neutralizing antibody to LIF in a procedure similar to that described for KL (Nilsson *et al*., 2002). Similarly, applying the same methodology, ovary culture in absence or presence of a factor and its function-blocking antibody, the stimulatory effect on primordial to primary transition was demonstrated for the factors: 1) basic fibroblast growth factor [bFGF; from FGF family with several roles in development: cell proliferation, migration and differentiation (Ornitz and Itoh, 2001)] and is produced in the oocyte (Nilsson and Skinner, 2001); 2) Bmp4 (growth factor from the TGF-β family member), produced by theca and stromal cells, which also was found important for follicle survival (Nilsson and Skinner, 2003); 3) keratinocyte growth factor [KGF; a fibroblast growth factor member that stimulates epithelial cell proliferation (Rubin et al. 1989)] is also produced by precursor-theca, theca and stromal cells (Kezele *et al*., 2005) and 4) platelet-derived growth factor (PDGF; a growth factor),

interestingly, appears to be produced by the oocyte (Nilsson *et al*., 2006). Bmp7 (also a growth factor member of the TGF-β superfamily), compared to vehicle, when injected into the ovarian bursa cavity, was also shown to activate primordial follicles and subsequent transition to primary follicle, (Lee *et al*., 2001).

The presence of three oocyte specific genes was also shown to be essential for primordial to primary transition: newborn ovary homeobox-encoding gene (Nobox) (Suzumori *et al*., 2002; Pangas *et al*., 2004), *Sohlh1*and *Lhx8* (Pangas *et al*., 2006). Nobox, as the name indicates, is a homeobox gene consequently involved in regulation of development and is expressed in oocytes of primordial, primary and growing follicles (Suzumori *et al*., 2002). Studies in deficient mice showed an arrest in follicle growth at primordial stage and a total loss of germ cells by day 14 after birth (Rajkovic *et al*., 2004). Nobox appears to regulate other important oocyte specific genes such as Oct4 and growth differentiation factor-9 (GDF9; from TGFβ superfamily) (Rajkovic *et al*., 2004). Both Sohlh1 and Lhx8 are transcriptional factors, which are expressed in germ cell clusters and oocytes of primordial follicles (Pangas *et al*., 2006). Analysis of female mice lacking the Sohlh1 gene revealed that primary follicles never form and complete depletion of germ cells occurs around 3 weeks post-birth (Pangas *et al*., 2006). A very similar pattern was encountered for Lhx8 deficient females and because the microarray analysis of Sohlh1$^{-/-}$ ovaries showed a dramatic downregulation of Lhx8, this may be indicative that Sohlh1 is a major regulator of genes involved in folliculogenesis (Pangas *et al*., 2006). Thus, these three genes are crucial for early oogenesis and folliculogenesis, particularly in primordial follicle activation. Figure 7 summarizes the factors involved in primordial follicle activation.

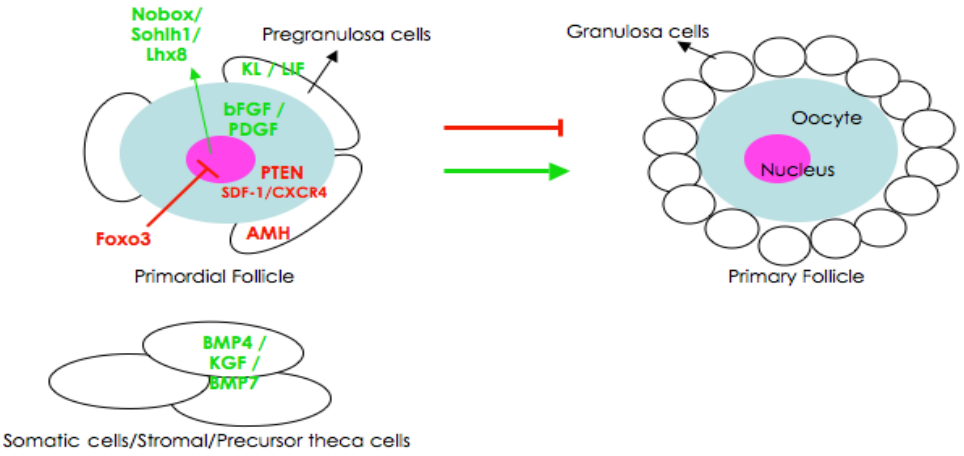

Figure 7. Schematic representation summarizing the inhibitory and stimulatory factors involved in the primordial to primary follicle transition.

Even though, FSH and/or LH influences some of these factors later in folliculogenesis, the transition from primordial to primary follicle is independent of gonadotropins (Buccione *et al*., 1990; Fortune, 2003). The previously described inhibitors and promoters of primordial follicle activation, produced by either oocyte, granulosa or theca cell precursors, reinforce the importance of cell-to-cell communication and illustrate that primordial follicle activation is a highly coordinated process (Albertini and Barrett, 2003; Skinner, 2005; Hutt *et al*., 2006b). Oocyte and surrounding granulosa cells are coupled to each other throughout folliculogenesis by gap junctions, which are the most important junctions within the ovary (Anderson and Albertini, 1976; Buccione *et al*., 1990; Albertini and Barrett, 2003). Gap junctions are intracellular membrane channels that allow sharing of small molecules between adjacent cells (Anderson and Albertini, 1976; Kidder and Mhawi, 2002). These are present in the mouse ovary as early as 17 days post coitum (Mitchell and Burghardt, 1986). The gap junction consists of groups of 6 protein subunits – connexins - joined together to form a channel, the connexon which is the functional unit of the gap junction; the end of the connexon from one cell docks with the end of the connexon from the adjacent cell to form the gap junction channel (Bruzzone *et al*., 1996). In the mouse ovary, several gap junction proteins have been identified, including connexins (Cxs) 37 and 43, which were demonstrated to be fundamental in folliculogenesis (Kidder and

Mhawi, 2002). Connexin 37 is exclusive to the interface between oocyte and granulosa cell. When Cxs 37 is absent, follicle growth arrests at the preantral stage (Type 4-see box 3) and although there is some growth of the oocyte it cannot initiate meiotic maturation (Simon *et al*., 1997; Carabatsos *et al*., 2000b). Connexin 43 is predominantly expressed in granulosa cells. In mice, targeted mutation of cx43 is lethal. Moreover, Cx43 is involved early in development since a reduced number of germ cells arrive to the genital ridges after Cx43 deletion (Juneja *et al*., 1999; Granot *et al*., 2002). When neonatal ovaries were cultured and/or grafted into the kidney capsule of adult females, folliculogenesis was arrested at the primary stage (Juneja *et al*., 1999; Ackert *et al*., 2001). Together these results underscore the importance of communication between granulosa cells and the oocyte. Cx37$^{-/-}$ mice support the transition from primordial to primary follicle, however Cx37 must be expressed for subsequent oocyte maturation and continued folliculogenesis (Liu *et al*., 2006). On the other hand, Cx43 has an earlier role and is required for connections between granulosa cells in order for these to proliferate, form additional granulosa cell layers and support continued follicle growth (Granot and Dekel, 2002; Gittens and Kidder, 2005; Teilmann, 2005; Simon *et al*., 2006).

Like females lacking Cx43, females of GDF9 null mice exhibited a block in folliculogenesis at the primary follicle stage (Dong *et al*., 1996). GDF9 is a member of the TGFβ super family expressed only in the ovary (McPherron and Lee, 1993) and found to be exclusively expressed in oocytes beginning in primary follicles and persisting to later developmental stages (McGrath *et al*., 1995). Interestingly, in hamster GDF9 seems to be essential for primordial follicle formation and granulosa cell differentiation (Wang and Roy, 2006). It was shown that GDF9 stimulates granulosa cell proliferation (Vitt *et al*., 2000), which might explain the arrest in folliculogenesis of GDF-9$^{-/-}$ female mice. Aberrant oocyte-granulosa cell interaction was found in these animals, indicating the involvement of the oocyte in somatic cell

proliferation, and reinforcing the importance of bidirectional communication between oocyte and granulosa cells and GDF9 paracrine actions in the ovary (Carabatsos *et al*., 1998; Elvin *et al*., 1999a; Elvin *et al*., 1999b). Moreover, it has been demonstrated that the impairment in folliculogenesis in $Cx43^{-/-}$ ovaries is in part because the granulosa cells cannot respond adequately to oocyte-derived GDF9 signals (Gittens *et al*., 2005).

Bidirectional communication is of vital importance to oogenesis and folliculogenesis, as has been shown using GDF9- and Cx43-deficient mice and reviewed by Eppig (2001) and Matzuk and colleagues (2002). The fundamental structures that provide this connection are the transzonal projections (TZPs), which are extensions from granulosa cells to the oocyte surface that establish and maintain the physical contact between these two cell types (Anderson and Albertini, 1976; Albertini and Rider, 1994; Motta *et al*., 1994; Albertini and Barrett, 2003). At the end of the TZP, where granulosa cells connect to the oocyte surface, are gap junctions (Anderson and Albertini, 1976; Motta *et al*., 1994). Interestingly, gap junctions at the end of TZPs are heterotypic composites between Cx43 and Cx37 (Kidder and Mhawi, 2002; Albertini and Barrett, 2003; Teilmann, 2005). Disruption of these gap junctions was found to be detrimental to folliculogenesis, as seen in both Cx37 and GDF9 null mouse models although the relationship between these oocyte specific genes has not been fully evaluated (Carabatsos *et al*., 1998; Carabatsos *et al*., 2000a). Cytoskeletal components of TZPs include both actin-filaments (Act-TZP) and microtubules (MT-TZP), both of which mediate shape and motility of the TZP (Albertini and Rider, 1994; Albertini *et al*., 2001; Navarro-Costa *et al*., 2005). Paracrine and hormonal regulation has been suggested as roles of TZPs for the vectorial secretion and/or uptake of signaling molecules at the oocyte-granulosa interface (Albertini *et al*., 2001; Combelles *et al*., 2004). These connections are already present in primordial follicles and continue

throughout folliculogenesis (Motta *et al*., 1994; Albertini and Barrett, 2003; Teilmann, 2005). The persistence of a coordinated communication network between oocyte and the surrounding somatic cells ensures ovulation of a healthy oocyte that is ready to be fertilized, the ultimate goal of folliculogenesis and oogenesis (Albertini *et al*., 2001; Eppig, 2001).

2. OOCYTE HYPERTROPHY: FROM PRIMARY TO MULTILAYERED FOLLICLES

The transition from primordial to primary follicle is prolonged to accommodate the growth phase of oogenesis. Hypertrophy of the oocyte is commensurate with a slow rate of granulosa cell proliferation when the follicle forms a second layer around the oocyte in secondary follicles (Fig. 6D). This protracted proliferative phase increases granulosa cells to six or seven layers in the pre-antral stage (Gougeon, 1996; Fortune, 2003). In the mouse, the appearance of a second layer of granulosa cells is accompanied by zona pellucida formation (Braw-Tal, 2002).

Numerous studies have been done in preantral follicles identifying factors responsible for follicle and oocyte growth, but the notion that the oocyte is the driving force for this event has been gaining acceptance (Eppig, 2001; Fair, 2003). In addition to the recognition of GDF9 as an oocyte specific factor, essential for follicular progression further than primary stage (Dong *et al*., 1996), another oocyte specific factor was discovered independently by two laboratories. It is either called bone morphogenetic protein-15 (BMP-15), due to its similarities to the BMP - family (Dube *et al*., 1998), or GDF9b because of its close homology to GDF9 (Laitinen *et al*., 1998), (herein referred to as BMP-15). BMP-15 is located on the X-chromosome and has an expression pattern very similar to GDF9 being predominantly in oocytes from primary follicles through ovulation in mouse (Dube *et al*., 1998), and rat (Otsuka *et al*., 2000). However, it appears that in some species GDF9 mRNA is present in primordial follicles, particularly in sheep (Galloway *et al*., 2000), cattle (Bodensteiner *et al*., 1999) and human (Aaltonen *et al*., 1999), implying that GDF9 synthesis precedes that of BMP-

15. This difference in species-specific expression patterns is interesting and may reflect local signaling requirements in monovular versus litter bearing (multiovular) animals. For example, studies with BMP-15 mutant mice revealed that null females are subfertile, with decreased ovulation and fertilization rates (Yan *et al*., 2001), but in sheep null BMP-15 females mimic the GDF9 null mouse phenotype in demonstrating follicular arrest at the primary stage (Galloway *et al*., 2000). At least in mice, this indicates that BMP-15 is more important in later stages of folliculogenesis, whereas GDF9 is needed earlier (Yan *et al*., 2001). Both factors were proven essential in sheep (Juengel *et al*., 2002), and their cooperative effect confirmed when recombinant ovine GDF9 and/or BMP-15 were shown to regulate the proliferation of granulosa cells in rat and ruminants (McNatty *et al*., 2005a; McNatty *et al*., 2005b). Furthermore, it was recently suggested that this cooperation is done through BMP – receptor II (Edwards *et al*., 2007). Interestingly, GDF9 and BMP-15 interact with KL (produced in the granulosa cells) controlling granulosa cell proliferation (Joyce *et al*., 2000; Otsuka and Shimasaki, 2002; Thomas and Vanderhyden, 2006). These growth factors have antagonistic roles: GDF9 inhibits KL expression in granulosa cells (Joyce *et al*., 2000; Wu *et al*., 2004), while BMP-15 acts as an activator of KL expression in granulosa cells, which in turn inhibits BMP-15 in a negative feedback loop (Otsuka and Shimasaki, 2002; Hutt *et al*., 2006a). At the same time KL from the granulosa cell seems to be the theca cell "organizer", inducing interstitial cell recruitment to form the theca layer (Parrott and Skinner, 2000). Evidence for GDF9 inhibition of KL comes from GDF9 null mice that demonstrate up-regulation of both KL and inhibin (Elvin *et al*., 1999a). Surprisingly, follicles of mice with a double knock-out (KO) for GDF9 and Inhibin-α developed to multilayered stages before ovarian tumors appeared. This indicates that granulosa cells proliferate without both factors, and that the up-regulation of inhibin in GDF9 null alone is responsible for preventing granulosa cell proliferation since its absence is sufficient to promote proliferation (Wu *et al*., 2004). Several models have

been proposed to explain the tight coordination between oocyte growth and granulosa cell proliferation, a time during oogenesis when many essential features of developmental competence are acquired.

One such model invokes coordinate KL and activin up-regulation that drives functional progression during folliculogenesis (Wu et al. 2004). Like activin, inhibin is a member of the TGF-β family and both were initially identified from follicular fluid through their ability to regulate FSH secretion *in vitro*; inhibin inhibits and activin stimulates FSH production (Ying, 1988; Knight and Glister, 2001; Knight and Glister, 2003; Lin *et al*., 2003). Follistatin, another component isolated from follicular fluid, is a single-chain glycoprotein with homology to α- and β-subunits of the inhibin/activin family that inhibits FSH production (Ueno, 1987). The regulatory action of follistatin over FSH comes from its capacity to bio-neutralize activin due to high affinity binding to this factor (Nakamura *et al*., 1990; de Kretser *et al*., 2002). These three regulators are expressed in granulosa and theca cells of antral follicles and in luteinized granulosa cells, having a weak expression in smaller follicles (Roberts *et al*., 1993). In fact, it was suggested that activin from bigger follicles stops smaller follicles from growing (Mizunuma *et al*., 1999). Evidence that these are important factors in the gonadotropin-dependent phase of folliculogenesis includes the facts that there is a 50% reduction in inhibin, and significantly reduced expression of activin in FSHβ deficient mice (Burns *et al*., 2001). These mice are infertile arresting follicular development prior to *antrum* formation (Kumar *et al*., 1997). FSH action is mediated by receptors in the granulosa cell membrane, and in accordance with FSH action of inducing antral follicle formation, female mice with no FSH-receptor (FSHr) gene also exhibited a block at pre-antral follicle stage and were infertile (Dierich *et al*., 1998; Abel *et al*., 2000). In these animals both inhibin and activin, were undetectable in serum and in gonads (Abel *et al*., 2000). Insulin-like growth factor-1 (IGF-1) has been described as having a similar function as activin and regulates FSHr, since it was shown to increase FSHr in granulosa cells (Zhou *et al*., 1997). Previously,

it was demonstrated that IGF-1 is selectively expressed in granulosa cells of healthy small pre-antral and antral follicles, and in the mural and cumulus cells of pre-ovulatory follicles (Oliver *et al*., 1989). The absence of the IGF-1 gene has a similar phenotype in the ovary as the loss of FSH; folliculogenesis does not proceed further than large pre-antral stage, and FSHr expression is significantly reduced (Zhou *et al*., 1997). Another suggested regulator of FSH in small pre-antral follicles is AMH, previously shown to inhibit primordial follicle activation (Durlinger *et al*., 1999). AMH action towards FSH was investigated due to the increased number of preantral and small antral follicles found in the ovary of adult AMH null mice despite the low level of FSH in serum (Durlinger *et al*., 2001). Results suggested that AMH has an inhibitory effect on FSH, balancing preantral follicle growth with FSH (Durlinger *et al*., 2001). These elaborate feedback and feedforward mechanisms likely evolved to support the growth phase of oogenesis through the symbiotic relationship of oocytes and granulosa cells.

Interestingly, FSH appears to serve as a master regulator of oocyte-granulosa cell communication as it has been shown to be capable of reversibly inducing TZP retraction (Albertini *et al*., 2001; Combelles *et al*., 2004). As emphasized previously, TZPs are important regulators of oocyte-granulosa cell communication, and the fact that the high number of TZPs observed in oocytes from FSH deficient females was significantly reduced with the addition of exogenous FSH is indicative of this coordination (Combelles *et al*., 2004). This coordination also implicates FSH action in maintenance of oocyte growth since oocytes depend on the metabolism of the granulosa cells for most of its macromolecular synthesis (Plancha *et al*., 2005).

The fact that granulosa cells in pre-antral follicles synthesize inhibin/activin, follistatin, and AMH and are capable of responding to FSH at a critical juncture in oogenesis further supports the importance of mutual signaling (McNatty *et al*., 2007). See figure 8 for a schematic simplification of the factors involved in multilayered follicle formation.

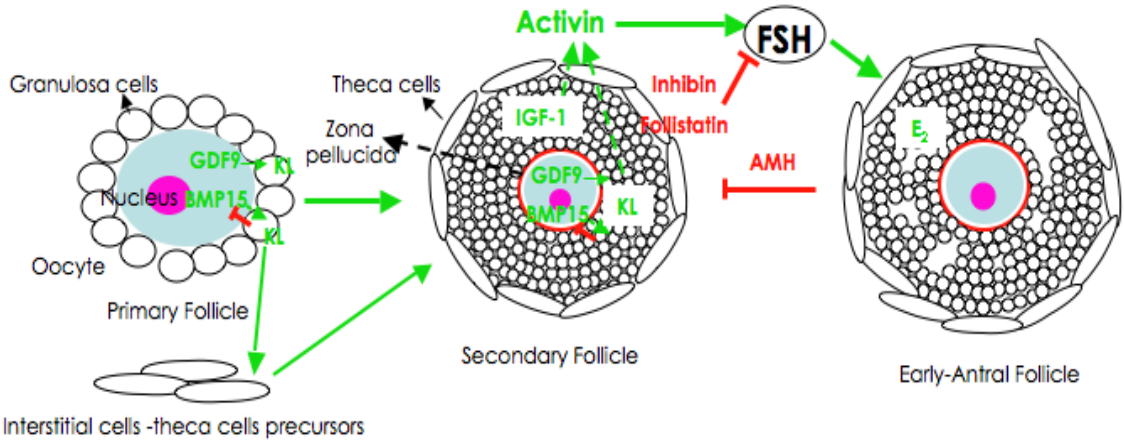

Figure 8. Schematic drawing of factors involved in multilayered follicle and early fluid-filled cavity formation.

3. ANTRUM FORMATION: ANTRAL FOLLICLES AND OVULATION

The continuous proliferation of granulosa cells leads to the production and accumulation of fluid forming small cavities within the layers – early antral follicles (Fig. 6E); eventually the cavities coalesce to become one big fluid filled cavity, the antrum – antral follicles (Fig. 6F) (Peters and McNatty, 1980). In antral stage depending on their location granulosa cells can be either *cumulus* cells, if closely surrounding the oocyte, or mural cells, if adjacent to the follicular wall (Peters and McNatty, 1980).

Even though small pre-antral follicles are responsive to FSH this hormone is not necessary for granulosa cell proliferation and follicle growth (McGee *et al.*, 1997). However the second phase of folliculogenesis is hormone dependent involving a complex process essential to the primary goal – ovulation of a fertilizable oocyte. Granulosa cell responsiveness to FSH marks the initiation of the second phase of folliculogenesis and it is dependent on FSHr, which is regulated by IGF-1 (Zhou *et al.*, 1997). In addition to IGF-1, estrogen acts in a supporting role to FSH in granulosa cell proliferation (Richards, 2001; Britt and Findlay, 2002). Estrogen is an endocrine steroid hormone produced by the granulosa cells, which also enhances FSH and induces granulosa cell differentiation (Britt and Findlay, 2002; Drummond, 2006). Estrogen is the product of the steroidogenic pathway (steroid biosynthesis through progressive carbon loss from

cholesterol) to estradiol-17β (E_2); higher amounts of E_2 are the major feature of the dominant follicle(s) (Hinshelwood *et al*., 1994; Baker and Spears, 1999; Drummond, 2006). Like every molecule, estrogens act through their receptors- estrogen receptors (ER) and the ovary has two subtypes ERα and ERβ. Despite the latter's greater abundance in the granulosa cells, the KO female mouse for ERα is infertile, with an arrest in folliculogenesis at antral development. On the contrary, ERβ null mice are subfertile (Couse *et al*., 1997; Krege *et al*., 1998; Couse and Korach, 1999) indicating that estrogen binds more ERα for proliferation (granulosa cells), and ERβ is probably used more in differentiation to stop proliferation and initiate necessary changes for ovulation (Britt and Findlay, 2002). The last step in E_2 production is catalyzed by aromatase, a member of the family of genes known as cytochrome P450 (Hinshelwood *et al*., 1994). When aromatase is not present, ovaries exhibit a phenotype similar to the ERα KO, follicular arrest at antral stage, indicative of the importance of both in folliculogenesis (Fisher *et al*., 1998). Aromatase is also regulated by FSH as demonstrated by the more than 6-fold decrease of P450 aromatase mRNA in the ovary of FSH-β KO females (Burns *et al*., 2001). Binding of FSH to its receptor in granulosa cells activates the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway, as well as other kinases such as serum and glucocorticoid-induced kinase (Skg) and PKB (protein kinase B)/Akt, downstream targets of the IGF-1/PI3K/phosphatidylinositol-dependent kinase 1 (PDK1) pathway (Gonzalez-Robayna *et al*., 2000; Richards, 2001). In agreement is the fact that in the FSHβ KO ovary Skg is significantly reduced (Burns *et al*., 2001). Interestingly, Skg has been implicated in proliferation of granulosa cells as well as their terminal differentiation into luteal cells (Alliston *et al*., 2000). Evidence for this FSH-cAMP/PKA pathway induced proliferation was demonstrated by studies on cyclin D2 null females, which have impaired folliculogenesis and no ovulation occurs due to abnormalities in granulosa cell growth (Sicinski *et al*., 1996). As a downstream effector in this pathway, IGF-1-induced

granulosa cell proliferation and stimulates LH receptors (LHr) in granulosa and theca cells (Magoffin and Weitsman, 1994) underscoring the importance of IGF-1 and E_2 interaction with FSH in folliculogenesis, initiating the period when granulosa and theca cells are responsive to LH (Richards, 2001; Richards *et al*., 2002). The key role of LH in folliculogenesis is evident in the study of mice with LH β-subunit disruption where no ovulation and no *corpora lutea* (CL) occurred (Ma *et al*., 2004). In addition, females lacking LHr develop early antral follicles, but no pre-ovulatory follicles or CL (Lei, Mishra *et al*., 2001; Zhang *et al*., 2001). Therefore, it's not surprising that LH KO females have low serum levels of E_2 and the steroid progesterone (Ma *et al*., 2004), which is produced by the theca and luteal cells under LH influence (Drummond, 2006). In normal mice, E_2 production by granulosa cells in response to FSH and LH induces a massive wave of proliferation among the granulosa cell population resulting in the formation of large pre-ovulatory follicles (Hirshfield, 1991; Robker and Richards, 1998). Once granulosa cells have acquired LH responsiveness (via LHr), theca cells produce increasing amounts of androgens (cholesterol sub-products prior to estrogens), the LH surge happens and the pre-ovulatory follicle activates a cascade of signalling proteins, which induce major transformation of follicular cells. They stop proliferation (cell cycle) and terminal differentiation (luteinization) begins, oocytes resume meiosis and ovulation occurs (Richards *et al*., 2002; Mehlmann, 2005).

Throughout follicle growth, the oocyte is arrested in meiotic prophase-I. Before ovulation, meiosis resumes in response to LH (Mehlmann, 2005). However only fully-grown oocytes are able to resume meiosis, therefore follicular growth is accompanied by oocyte growth. As the follicle grows and matures, the oocyte volume increases greatly due to massive RNA storage and complex organelle reorganization (Wassarman and Albertini, 1994). Additionally, specific oocyte structures such as the zona pellucida (deposition of the glycoproteins ZP1, ZP2 and ZP3) and cortical granules are

formed (Wickramasinghe and Albertini, 1993; Wassarman and Albertini, 1994). The zona pellucida is important not only in fertilization as shown by KO analysis of ZP1 that had reduced litters (Rankin *et al*., 1999), but also in granulosa-oocyte communication as indicated by genetic disruption of ZP2 and ZP3, which results in mice that ovulate but no zona is present and dramatic alterations are seen at the interface of oocyte and granulosa cell (Rankin *et al*., 1996; Rankin *et al*., 2001). Cortical granules, on the other hand are small organelles that localize near the oolema of mature oocytes, expelling their contents into the perivitelline space following fertilization and alter ZP properties to block polyspermy (Wassarman and Albertini, 1994).

The oocyte acquires meiotic resumption capacity when follicles become antral (Mehlmann, 2005). This acquisition involves structural rearrangements in the oocyte cytoplasm and nucleus, including cytoskeleton organization. Cytoplasmically, in the meiotic competent oocyte short microtubules (MTs) are enucleated from phosphorylated centrosomes while the incompetent oocyte exhibits long arrays of MTs and non-phosphorylated centrosomes (Wickramasinghe and Albertini, 1992). In general oocyte cytoplasmic maturation is accompanied by loss of MTs and acquisition of multiple microtubule organizing centers (MTOCs) (Mattson and Albertini, 1990). Regarding nuclear rearrangements, incompentent oocytes are characterized by a non-surrounded nucleolus (NSN) chromatin configuration, whereas competent oocytes due to progressive chromatin condensation display a surrounded nucleolus (SN) (Mattson and Albertini, 1990; Albertini *et al*., 2003). However, in both competent and incompetent oocytes the nucleus is named germinal vesicle (GV). The first of two steps in meiotic acquisition is GV breakdown (GVBD), nuclear envelope breakdown with consequent progression to metaphase I (MI, reduction division), secondly the oocyte gains the capacity to move from MI into MII (metaphase II) (Wassarman and Albertini, 1994). In the mouse, briefly before GVBD MTOCs are activated in the vicinity of the chromosomes and

MTs are stabilized and become progressively organized into a bipolar spindle around the chromosomes (Brunet and Maro, 2005).

B. How oogenesis presupposes embryogenesis

1. The maternal legacy in organisms with distinct reproductive strategies

Central to an understanding of oogenesis in an organismal context is recognition of the physiological mechanisms that establish the timing of this process. Nutritional status and metabolic stability are two important factors that organisms use to initiate and maintain the process of oogenesis. Thus it is not uncommon that animals encountering environmental or seasonal restrictions in diet opt to stop or delay oogenesis until appropriate nutritional requirements can be met to support what is often a metabolically taxing process. This basic tenet is the core of oogenesis that defines the role of hypertrophy and storage of organelle precursors that are called upon to support the metabolic demands of embryogenesis. In general, the embryo reaches a stage of self-sufficiency either by having reached a level of feeding competency or by establishing a maternal source of nutrition through the placenta. The second major factor that links oogenesis to embryogenesis is the ability to establish continuity of organelles from one generation to the next. Here the relationship between genes and the environment has taken on increased significance for future studies.

2. Maternal effectors: from genes to environments

Maternal effectors play critical roles in oogenesis. While this has been appreciated for many years from studies on maternal effector genes in flies and worms, the significance of this to mammalian oogenesis has only recently been demonstrated. As shown in Table 1, there are now many gene deletion models in the mouse that illustrate both classical examples of maternal effector genes (MATER, ZAR1, Dmnt1O) and genes active in either the oocyte or surrounding somatic cells that are required for

progression of oogenesis or folliculogenesis. The emerging trend from a compilation of phenotypes implies a form of oocentric regulation at the onset of oocyte growth that is then subject to mutual germ line somatic cell feedback control that invokes both local paracrine elements as well as systemic interactions between the ovary and various somatic tissues (Hutt and Albertini, 2007). Thus, maternal effectors for oogenesis are both genetic and absolute in terms of specific gene products that are required to initiate and sustain embryogenesis. Given the dependency on somatic control in mammals like the mouse, there is every reason to believe that epigenetic factors profoundly influence oogenesis. Recent studies on environmental toxins have reinforced this concept in a rat model (Hutt *et al*., 2008).

3. Modifiers of developmental competence in mammals

Developmental competence in mammalian oocytes refers to the well-established fact that discrete transitions in the physiology of the oocyte define the acquisition of a functional capacity. For example, the primordial to primary oocyte transition initiates an overt hypertrophic condition without cell cycle resumption from meiosis 1 arrest and therefore is analogous to the G2 stage of the somatic cell cycle (Wickramasinghe and Albertini 1993). As noted above, this transition is modified by many factors of oocyte and granulosa cell origin (see Table 1). This is followed sequentially by the acquisition of meiotic competence, the competence to initiate appropriate fertilization responses like the cortical reaction and exit from meiosis 2, and the competence to affect early mitotic cell cycles in the embryo. Notably, each of these competencies is acquired at specific stages of folliculogenesis. Thus, modifiers of a very diverse nature are likely to impact the developmental competencies of an oocyte. Current work in this area is focused on maternal modifiers that include age, hormonal or nutritional status of females as well as direct or indirect consequences that result from environmental exposures [e.g. bisphenol A

(BPA) (Susiarjo *et al*., 2007)] or following therapeutic interventions for fertility or cancer. The conclusion here is that there are high risks in organisms such as mammals that exhibit prolonged segments of their pre and post-pubertal lifespan that modifiers of oogenesis will impact the developmental competence of oocytes and embryos without necessarily interfering with the completion of pregnancy. Understanding and defining those segments of female lifespan that are at greatest risk for genetic or epigenetic modification of oogenesis is a major and timely challenge that lies ahead (Bromfield *et al*., 2008).

C. Strengths and Limitations of Animal Models

1. Intrinsic vs Extrinsic Control

Comparative physiology provides a useful forum for validating and extending the utility of animal models for understanding human disease. The field of oogenesis has benefited enormously from the use of animal models, but parallel advances in humans and mammals other than the mouse call into question the translational significance of this line of study. Specifically, the relative contribution of factors intrinsic or extrinsic to the process of oogenesis that ensure oocyte developmental competence has been largely ignored. What might these factors be? Nutrition, environmental exposure, and reproductive lifespan have already been implicated as extrinsic factors that would directly or indirectly influence ovarian physiology. Oocyte intrinsic gene expression has similarly been identified via gene targeting or RNA knockdown strategies. But the confluence of these two broad categories of regulatory principles has yet to be fully appreciated and may reside in the physiological regulation between the germ line and somatic cells with which the oocyte is in direct contact. It is with this in mind that a comparative analysis may be helpful in defining the utility of animal models.

2. Flies, worms and mice at the front edge

Figure 9 serves to illustrate the extreme variability in the basic design pattern at the interface of oocytes with follicle cells. Highly seasonal spawners like marine invertebrates tend to modify the coelomic epithelium into extensive folds of germinative potential accounting for much of an animals' metabolic energy for egg production. A role for follicle cells emerges in organisms that invest in vitellogenesis more selectively by producing relatively fewer developmentally competent oocytes and establishing defined physical inputs from the surrounding somatic cells (Fig. 9B). The amplification of cell contact between follicle cells and oocytes is a hallmark of virtually all-eutherian mammals (Fig. 9C and D). Enhancement of the communication opportunities between the soma and germ line is not necessarily a carry over from the need to provide yolk precursors to the growing oocyte and instead seems to be a reflection of the growing need to monitor and promote a sensing system that titrates both somatic and germ line health. If such a concept holds up, it will be important to consider the varying demands for oogenesis in model systems that use stem cells to propagate new oocytes (flies, worms), or that deploy relatively short prepubertal stages to establish a small fixed supply of oocytes that would last the entire reproductive lifespan (mouse). These model organisms remain mainstays in the field of oogenesis research and will continue to contribute invaluable genomic and epigenomic information with which to further explore the problem of oogenesis. They may not, however, be the most opportune for understanding human disease.

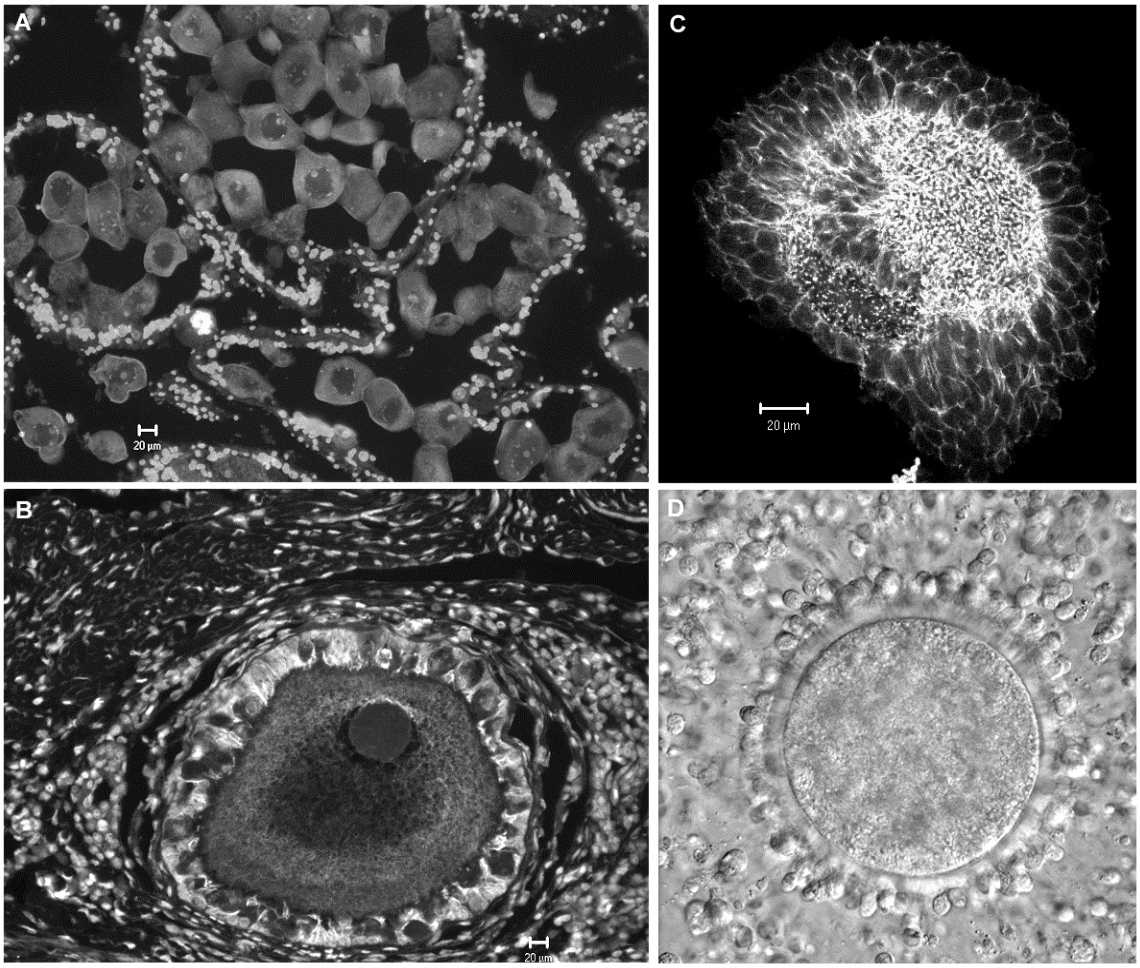

Figure 9. Micrographs depicting variety of oocyte somatic cell interactions observed in (A) surf clam, (B) ray, (C) canine, and (D) bovine. Note the limited germ cell contact in surf clam (A), where oocyte clusters are released from outpocketings of the coelomic epithelium; in highly vitellogenic species like the ray (B), a single layer of follicle cells labeled with acetylated tubulin antibodies is depicted in which apical projections approximate the oolemma. In mammals (C and D), subsets of granulosa cells are anchored to the zona pellucida that project many thousands of actin-containing transzonal projections (Act-TZPs) making direct physical contact with the oolemma. (C) Confocal projection of canine cumulus oocyte complex before ovulation stained with rhodamine phalloidin to illustrate the density of f-actin projections at the zona surface. (D) DIC image of a bovine cumulus complex after ovulation in which many projections are retained within the zona pellucida that is surrounded by a single layer of granulosa cells known as the corona radiata. Scale bars 20µm.

3. Future Model Systems

From the above discussion, it is clear that if alternative model systems were available for purposes of clinical translation, a discrete boundary could be drawn between the investigative utility of model systems and the pressing needs emerging from the fields of human stem cell and ART research. In this regard, the shortcomings of material shortage, ethical issues, and

experimental tractability have often been raised as impairments to research on the biology of oogenesis in humans. This landscape is changing. Opportunities to store ovary, oocytes, and embryos have increased as cryopreservation technology has advanced this field from the mouse to human materials. Using unfertilized parthenogenetically activated oocytes bypasses some ethical concerns but may ironically add a new twist to this debate in validating the Immaculate Conception. And finally, bioinformatics resources and approaches are supplying the necessary foundation for asking direct questions about human oocytes that will aid in understanding the defects and deficiencies that underscore female reproductive health.

D. Conclusions

This review will hopefully have served two purposes: 1) to highlight the complexities inherent in oogenesis that need to be solved to understand quality egg production, and 2) to draw timely comparisons between various animal models with respect to their utility in addressing the problem of maternal inheritance and totipotency. Oocytes in mammals are rare and highly specialized cells whose livelihood holds the fate of future generations. They are also very culpable cells subject to the whims and fancies of their somatic surrounds and as such are targeted for modification or destruction by lifestyle, misguided immune regulation, environmental toxins, hormones, and medications that are being used increasingly in immune suppression or chemotherapy. Model systems will continue to provide a wealth of information bearing on fundamental aspects of the process of oogenesis but extrapolations to mechanisms that bear on human or animal health will require a species-specific approach. Thus, protecting the female germ line will necessitate that experimentalists, clinicians, public health specialists, and ethicists synergize their efforts in devising acceptable standards and approaches for research on the biology of human oocytes.

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1.3. OVULATION

Ovulation is the process by which a Graffian-follicle ruptures and releases a mature fertilizable oocyte surrounded by cumulus-cells. Follicle rupture occurs at the ovary surface in response to a surge of LH, which stimulates a cascade of events that end in oocyte release (Richards, 2001). LH acts on the granulosa cells, specifically increasing progesterone receptors (PR). Again, not surprisingly gene disruption has showed that several genes are involved in the process (Robker *et al*., 2000; Richards *et al*., 2002). Including progesterone receptor (PR), cyclooxygenase-2 (COX-2) (Lydon *et al*., 1996; Sicinski *et al*., 1996; Lim *et al*., 1997). Albeit progesterone is usually associated to establishment and maintenance of pregnancy, the inability of null PR females to ovulate demonstrated progesterone importance in the final step of folliculogenesis (Lydon *et al*., 1996). Concomitantly, COX-2 gene disruption produces a similar outcome, ovulation failure and even when some oocytes are recovered these have abnormalities and do not fertilize (Lim *et al*., 1997). COX-2 also known as prostaglandin endonuclease synthase (PTGS) is an enzyme involved in prostaglandin (PG) biosynthesis, which has fundamental function in inflammatory response, in this particular case ovulation (Lim *et al*., 1997; Sirois *et al*., 2004). It was shown that LH induces COX-2 expression (Sirois and Richards, 1992). Interestingly, there are evidences for oocyte control of COX-2 production; it was demonstrated that GDF9 at the time of the LH surge induces progesterone production in granulosa cells of pre-ovulatory follicles via prostaglandin E_2 (PGE_2) receptor (EP_2) pathway (COX-2/ PGE_2/EP_2) (Elvin *et al*., 2000; Joyce *et al*., 2001). Also involved in cumulus cells expansion due to problems encountered in mice lacking the gene for EP_2 (Hizaki *et al*., 1999). PGE_2 was

recently suggested to be one of local signalling molecules coordinating oocyte maturation, ensuring the ovulation of a fertilizable oocyte (Duffy *et al.*, 2010). On the other hand, progesterone LH surge-induced also promotes the expression of two other important proteases: A disintegrin and metalloproteinase with thrombospondin-like motives-1 (ADAMTS-1), and cathepsin L (a lysosomal cysteine enzyme) (Robker *et al.*, 2000; Doyle *et al.*, 2004). ADAMTS-1 is a member of the ADAMTS family of metalloproteinases necessary to ovulation as indicated by the higher number of anovulated oocytes in ADAMTS-1 null females (Shozu *et al.*, 2005), or in regulation of cumulus cells expansion hormone-induced (Shimada *et al.*, 2004). Cathepsin L showed hormone dependent expression in mouse and rat, present in granulosa cells of small and growing follicles with increased in pre-ovulatory follicles (Sriraman and Richards, 2004). Although its function is poorly known, a possible role could be remodelling of extracellular matrix, so important in ovulation (Sriraman and Richards, 2004). Recently, it was suggested that hypoxia-inducible factors (HIF's) are downstream regulators of PGR function (Kim *et al.*, 2009). HIF's mediate transcriptional responses to localized hypoxia, normal or tumour tissues, Kim and colleagues (2009) showed that if HIF is inhibited ovulation is blocked. The inhibition of HIF also induces suppression of Vegfa, Adamts-1, known regulators of ovulation (Kim *et al.*, 2009).

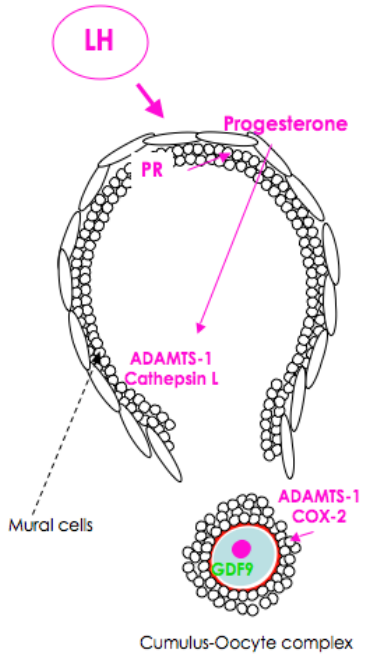

Figure 10. Simplified scheme describing the main factors involved in ovulation.

Figure 10 summarizes these events in a schematic simple way.

1.4. OBJECTIVES

The production of developmentally competent oocytes is probably the ovary's major goal. The full understanding of how the ovary and particularly the follicle pool is established and maintained is still not completely understood, hence it is pertinent its study. In addition, a better understanding of these processes will also be important towards several infertility situations and better Assisted Reproduction Technologies (ARTs). In this regard we analyse mouse ovary at different ages, from foetal to adulthood with the purpose of bringing an insight on the functioning of a complex organ such as the ovary. Using basic histology techniques allied to follicle and ovary culture, fluorescence and live imaging, or genetic manipulated mouse model, we hope to uncover ovarian hidden aspects and shed light on the functioning of this fascinating organ.

The main objectives of this thesis are to better understand:

1) Ovarian follicular reserve establishment, through identification of early germ cell loss and the cell death mechanisms involved;
2) Ovarian and follicular histogenesis, through detailed analysis of somatic-germ cell interplay;
3) Hormonal versus oocyte roles in establishing and maintaining the follicular reserve, through detailed analysis of appropriate mouse knockout models (FSHβ, GDF9, Nobox, and Sohlh1) and their differential somatic-germ cell interactions.

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Table 1 – Mouse mutation and phenotypes that affect oogenesis (early and late).

Gene mutated	Mouse knockout phenotype	Reference
Primordial germ cells – PGC's deficiencies		
B-cell lymphoma/leukaemia 2 (*Bcl2*)	Reduced survival of PGC's	Ratts *et al*., 1995
Bcl2-associated X (*Bax*)	Increased follicular endowment	Greenfeld *et al*., 2007
Bone Morphogenetic Protein 4 (*Bmp4*)	No PGC's	Lawson *et al*., 1999
Bone Morphogenetic Protein 8 (*Bmp8*)	Greatly reduced or no PGC's	Ying *et al.*, 2000
Caspase 4 (*Casp 4*; also known as *Casp11*)	Reduced number of oocytes at birth	Morita *et al*., 2001
Connexin 43 (*Gja1*)	Reduced PGC's	Juneja *et al.*, 1999
Dominant white spotting (*W*), encode for and c-kit	Improper PGC migration and germ cell deficiency	Buehr *et al.*, 1993 (*W*)
Follistatin (*Fst*)	Early germ cell loss, sex-reversal	Yao *et al.*, 2004
Octamer – 4 (*Oct4*)	Massive apoptosis of PGC's	Kehler *et al.*, 2004
Peptidyl-prolyl isomerase 1 (*Pin1*)	Impaired germ cell proliferation	Atchison *et al.*, 2003
Smad 5	Greatly reduced or no PGC's	Chang and Matzuk 2001
Still (*Sl*), encode for kit ligand (KL)	Improper PGC migration and germ cell deficiency	Mahakali Zama *et al*., 2005 (*Sl*)
Wnt4	Early germ cell loss, sex-reversal	Vainio *et al.*, 1999
Primordial follicle (POF) assembly deficiencies		
Aryl-hydrocarbon receptor (*AhR*)	Affects germ cell apoptosis – increased POF numbers	Robles *et al*., 2000
Dazla	Complete absence of follicles	Ruggiu *et al*., 1997
Factor in germline alpha (*Figl*α)	No POF assembly, complete germ cell loss by day 2	Soyal *et al*., 2000
Pre-antral follicles deficiencies		
Anti-Müllerian hormone (*Amh*)	Enhanced follicle recruitment	Durlinger *et al.*, 1999
Connexin 43 (*Gja1*)	Arrest in primary follicles	Ackert *et al.*, 2001
Connexin 37 (*Gaj4*)	Arrest at pre-antral stage of folliculogenesis and meiotically incompetent oocytes	Simon *et al.*, 1997 and Carabatsos *et al.*, 2000b
Estrogen receptor α (*Esr1* – ERKO)	Arrest of follicle growth at early antral formation	Couse *et al.*, 1997 and Dupont *et al*., 2000
Follicle stimulating hormone (*Fsh*β)	Arrested folliculogenesis prior to antral follicle formation	Kumar *et al.*, 1997
FSH receptor (*FshKO*)	Arrested folliculogenesis prior to antral follicle formation	Abel *et al.*, 2000
Follitropin receptor (*FORKO*), all FSH-R variants	No antral follicle formation, fewer germ cells	Danilovich *et al*., 2001 and Danilovich *et al*., 2004
Forkhead box L2 (*Foxl2*)	No secondary follicles, no granulosa cell differentiation	Schmidt *et al*., 2004 and Uda *et al*., 2004
Forkhead box O3 (*Foxo3*)	Rapid oocyte depletion due to POF recruitment	Castrillon *et al.*, 2003
Growth differentiation factor-9 (*Gdf9*)	Arrest at primary stage folliculogenesis; no theca layer and incompetent oocytes	Dong *et al.*, 1996 and Carabatsos *et al.*, 1998
Insulin-growth factor-1 (IGF-1)	Folliculogenesis arrest at multilayered follicles, no antrum formation	Zhou *et al.*, 1997

Gene mutated	Mouse knockout phenotype	Reference
Pre-antral follicles deficiencies (cont.)		
Lhx8	Early germ cell loss, no primary follicle formation	Pangas *et al.*, 2006
Newborn ovary homeobox (*Nobox*)	Deficient POF to primary transition, no germ cells by day 14	Rajkovic *et al.*, 2004
Sohlh1	No primordial to primary transition, early germ cell loss	Pangas *et al.*, 2006
Smad3	Impaired folliculogenesis, no antral follicles formed	Tomic *et al*., 2004
Ovulation and fertilization deficiencies		
A disintegrin and metalloproteinase with thrombospondin-like motives-1 (ADAMTS-1)	High rate of anovulation	Shozu *et al.*, 2005
Aromatase – CYP19 (ArKO)	No ovulation due to impaired folliculogenesis	Fisher *et al.*, 1998 and *Britt et al*., 2001
Cyclooxygenase-2 (COX-2)	Ovulation failure, no fertilization	Lim *et al*., 1997
Cyclin D2	Abnormal GC proliferation, oocytes do not ovulate	Sicinski *et al.*, 1996
Bone Morphogenic protein-15 (Bmp15)	Small litters – reduced ovulation and fertilization rates	Yan *et al.*, 2001
Estrogen receptor β (Esr2 – BERKO)	Reduced number of oocytes ovulated, small litters	Krege *et al.*, 1998
Inhibin α	Reduced number of oocytes ovulated, small litters	Matzuk *et al*., 1992
Luteinizing hormone (Lh)	Infertile, no ovulation	Ma *et al.*, 2004
LH-receptor (Lhr)	Antral follicles, but no ovulation	Lei *et al.*, 2001 and Zhang *et al.*, 2001
Progesterone receptor (PR)	No ovulation	Lydon *et al*., 1996
Zona pellucida 1 (ZP1)	Loose organization of zona pellucida – reduced litters	Rankin *et al.*, 1999
Zona pellucida 2 (*ZP2*)	Thin zona pellucida that disappears in late folliculogenesis and ovulated oocytes – infertile	Rankin *et al.*, 2001
Zona pellucida 3 (ZP3)	No zona pellucida and no cumulus expansion – infertile	Rankin *et al.*, 1996

CHAPTER # 2

Coordinated and developmentally regulated mechanisms of germ cell loss in the mouse ovary

(Patricia Rodrigues; Darlene Limback; Lynda McGinnis; Carlos E Plancha; and David F Albertini – *Reproduction(2009)* ***137****:709-720*)

ABSTRACT

In the perinatal ovary of most mammals, external and internal factors establish a primordial follicle reserve that specifies the duration of the reproductive lifespan of a given species. We analyzed the mechanism of follicle loss and survival in C57Bl6 mice using static and dynamic assays of apoptosis, autophagy, and ovarian morphogenesis. We confirm an initial loss soon after birth, when about 44% of the germ cells detectable at the end of the foetal period abruptly disappear. The observations that (1) few germ or somatic cells were apoptotic in newborn ovaries, (2) vitally stained organ cultures exhibit active extrusion of non-apoptotic germ cells and (3) germ-cell lysosome amplification occurs at birth suggested that additional mechanisms are involved in perinatal germ cell loss. Newborn mouse ovaries cultured in the pH sensitive dye lysotracker red (LTR) exhibit an increased incidence of acidified non-apoptotic germ cells when maintained in the absence, but not in the presence of serum, implying a role for autophagy in germ cell attrition. Inhibitors of autophagy, but not apoptosis, reduce germ cell acidification induced by serum starvation in ovary organ cultures and protein mediators of both autophagy and apoptosis are expressed at birth. From these findings we suggest that multiple perinatal mechanisms establish the primordial follicle reserve in mice.

INTRODUCTION

The transition from foetal to neonatal life is a critical phase of normal development in eutherian mammals during which organ systems adapt to post-parturition starvation. Many organ systems in newborns adjust progenitor cell density and function by engaging apoptotic and non-apoptotic mechanisms of programmed cell death (PCD) (Edinger and Thompson, 2004). There are three types of PCD. Type I or apoptosis, is characterized morphologically by nuclear condensation and fragmentation, cell shrinkage and membrane blebbing (Edinger and Thompson, 2004; Tilly, 2001). Type II or autophagy, is a lysosomal degradation pathway that involves formation of autophagic vacuoles during periods of massive cell elimination (Edinger and Thompson, 2004; Qu et al., 2007). Type III or necrosis, is characterized by plasma membrane breakdown causing an inflammatory reaction (Edinger and Thompson, 2004). Apoptosis is coupled to autophagy as a means to maintain tissue viability and energy homeostasis in developing tissues during the demanding neonatal phase in mammals (Kuma *et al*., 2004; Lum *et al*., 2005; Yu *et al*., 2004). This coupling occurs either via constitutive pathways, where lysosomes degrade the apoptotic bodies, or through an alternative pathway in which lysosomal proteases such as cathepsins, trigger apoptosis (Bursch, 2001; Guicciardi *et al*., 2004). Amongst the organ systems that exhibit profound cell loss at birth is the ovary, where it has long been recognized that massive female germ cell attrition postpartum precedes the establishment of a fixed follicle reserve that is progressively depleted during the reproductive lifespan (Qu *et al*., 2007; Reynaud and Driancourt, 2000; Tilly, 2001). Studies favouring a major role for germ cell apoptosis include those using ubiquitously targeted deletion of genes involved in Type I PCD. Accordingly, an increase or decrease in follicle numbers has been reported in postnatal mice bearing systemically targeted gene deletions for anti-apoptotic, or pro- apoptotic genes (Perez *et al*., 1999). Although apoptosis is believed to account for the bulk of germ

cell loss in foetal mouse ovaries (Coucouvanis *et al*., 1993; De Pol *et al*., 1997; Pepling and Spradling, 2001), other processes such as germ cell extrusion (Wordinger *et al*., 1990) and autophagy, also participate in pre and postpartum adjustments of germ cell numbers (Lobascio *et al*., 2007; Wordinger *et al*., 1990). Both apoptosis (Edinger and Thompson, 2004; Maiuri *et al*., 2007) and autophagy (Maiuri *et al*., 2007), lysosome-mediated PCD processes (Guicciardi *et al*., 2004), have been proposed to serve cooperatively during developmental transitions in other organs, but this prospect has yet to be studied during perinatal ovarian germ cell loss.

The present studies address several questions related to morphogenesis of the mouse ovary revealing a previously unappreciated level of complexity in programmed cell death that sets and maintains adequate numbers of follicles for reproductive function in mice without invoking replacement strategies. Our findings suggest that multiple mechanisms including autophagy mediate germ cell loss and are used in a coordinated and developmentally regulated fashion.

MATERIALS AND METHODS

Animals

Inbred C57Bl/6 mice (Charles River, or Taconic Farms Inc., Germantown, NY) used for these experiments were housed in a 14h light: 10h dark environment at constant temperature. Food and water was provided *ad libitum*. All animals were sacrificed by cervical dislocation. Mice were maintained and used in accordance with the policies of the University of Kansas Animal Care and Use Committee (protocol # 2007-1681).

Ovary collection and tissue preparation

Right and left ovaries from each animal were collected at embryonic (E15.5 and E19.5), prepubertal (P2, 6, 10, 12, 16, 20 days of age) and adult (P42, 100 and 150 days of age) stages. Five ovaries were collected at each stage from at least two separate litters. Intact bursa enclosed ovaries were

fixed in either Bouin's fluid (Sigma; follicle counts) or 2% paraformaldehyde (PFA, Sigma; immunohistochemistry), for 4-6h at room temperature and overnight at 4°C, respectively. Following fixation, ovaries were transferred to 70% ethanol and processed for paraffin embedding by standard techniques. For whole mount analysis, intact ovaries were fixed overnight in 2% formaldehyde microtubule stabilization buffer (MTSB-XF), (Messinger and Albertini, 1991) at 4°C and stored at 4°C in a blocking buffer as previously described until use.

Immunocytochemistry

For follicle counts 5µm sections were dewaxed and re-hydrated using conventional methods. Endogenous peroxidases were quenched in 0.3% hydrogen peroxide in methanol (5 minutes, room temperature). After three washes in Automation Buffer (BiØmeda-Fisher), slides were blocked for 30 minutes at room temperature in 9% goat serum (Zymed) containing 3% Bovine Serum Albumin (BSA, Sigma). Sections were labelled (overnight, 4°C) with polyclonal rabbit antibody against mouse vasa homolog (MVH, a cytoplasmic germ cell specific protein, gift from Dr. Noce) diluted 1:1200 in 1% BSA. Washed sections were then incubated with biotinylated goat anti-rabbit serum (Zymed; 1:200 in 1% BSA) for 30 minutes at room temperature, rinsed and incubated with Horseradish Peroxidase Avidin D (HRP; Vector; 1:500) for 10 minutes. Diaminobenzidine (DAB) was used as a substrate for HRP. Sections were counterstained with periodic acid-Schiff's reagent and Harris hematoxylin (Protocol) containing 4% acetic acid. Tissues were dehydrated, cleared and coverslipped using Permount (Fisher). Control slides were processed identically but primary antibody was omitted.

Follicle morphology and classification

Follicles were classified according to Pederson and Peters (Pedersen and Peters, 1968) and Myers et al. (Myers *et al*., 2004). Briefly, follicles were classified as primordial if the oocytes were partially or completely

surrounded by squamous granulosa cells (GCs), equivalent to Type 1-2 in the Pederson and Peters (1968) classification. Primary follicles were those exhibiting one complete layer of cuboidal granulosa cells, (Type 3-3b). In the transition from primordial to primary classification was made according to the predominant type of granulosa cells present. Secondary follicles were classified as all follicles having more than one granulosa cell layer and no visible antrum (Type 4-5). Follicles with a small antrum were designated early antral (Type 6) and antral follicles (Type 7) when the follicle had a single large antral space.

Follicle counting

Counts were made using a 40x Nikon objective in an Alphaphot 2 SY2 Nikon microscope. Follicles with an intact oocyte nucleus containing a dark nucleolus were counted in every second section and repeat scoring was unlikely because ~12µm germ cells in primordial and primary follicles would not be present in the third 8µm section used for sampling frequency in this study. For these follicle classes, final totals were derived by doubling the follicles recorded in every second section. However, for secondary to pre-ovulatory follicles this adjustment was omitted in order to limit over-representation (Liu *et al.*, 2002). The position of primordial follicles relative to the ovarian surface epithelium was also determined and these were scored as extra-ovarian (if in the bursal cavity) or intraepithelial (if associated with or subjacent to the epithelium).

Static and Dynamic Imaging of Germ Cell Loss

Programmed cell death was studied using a combination of methods to discriminate between apoptosis and autophagy in histological samples or ovarian whole mounts.

Apoptosis detection

TUNEL, i.e., terminal deoxynucleotidyl transferase-mediated dUTP nick end

labelling, was used to detect apoptosis following the manufacturer's protocol for the ApopTag Peroxidase *In Situ* apoptosis detection kit (Chemicon). For active Caspase-3 (1:100; R&D Systems cat. # AF835), and Poly (ADP ribose) Polymerase (cleaved-Parp form; 1:50; AbCam cat. # ab32064) paraffin sections of PFA fixed ovaries were dewaxed and microwaved (high, 15 minutes) in sodium citrate buffer (0.01M, pH 6.0) for antigen retrieval. After sections were washed and blocked they were incubated in primary antibody (see above). For fluorescent imaging, slides were exposed to Alexa-fluor goat anti-rabbit secondary antibodies (488 or 568; 1:800; Molecular Probes-Invitrogen; 37°C; 1h) rinsed (3X), incubated in Hoechst 33258 (1µg/ml; Polysciences Inc.) for nuclear staining and mounted in Prolong Anti-fade Reagent (Molecular Probes-Invitrogen). Control slides were prepared as above by omitting primary antibody.

Whole mount preparations of intact or fragments of ovaries fixed in MTSB-XF (Messinger and Albertini, 1991) were processed for confocal microscopy using the same primary and secondary antibodies noted above. These were diluted with wash solution and used with constant agitation for 24h at 4°C. Multiple wash steps (a total of 4h, several changes; 37°C) separated primary and secondary antibody incubations. Hoechst 33258 (nuclei) and Alexa 546 Phalloidin (f-actin; 1:100 dilution, Molecular Probes-Invitrogen) were used to define nuclear and cell boundaries. Ovaries were mounted on microscope slides in glycerol/PBS containing Hoechst 33258.

LysoTracker Red (LTR) is an aldehyde-fixable dye that concentrates in acidic membrane-bound intracellular compartments of living tissue, which has been adopted by Zucker and colleagues (Zucker *et al*., 1998) as an assay for programmed cell death in rodent tissues. We modified the protocol as follows: intact ovaries were incubated in 5µM LTR (Molecular Probes-Invitrogen) in MEM (Gibco-Invitrogen) for 1h to 2h at 37°C and 5% CO_2. After two washes in PBS the ovaries were fixed in a mixture of 4% PFA and 1% glutaraldehyde (Sigma) in PBS (2h; 37°C with shaking, followed by 4° C, overnight), rinsed twice in PBS and dehydrated in a series of

methanol/PBS solutions (50%, 70%, 95% and 100%- 2X; 15 minutes each). Tissues were then cleared in a 1:2 mixture of Benzyl Alcohol (Sigma) to Benzyl Benzoate (Sigma - BABB). From absolute methanol, tissues were exchanged through 50% and 70% BABB-methanol and finally pure BABB solution (2h each; at room temperature with constant agitation). For confocal microscopy, a single ovary was centrally positioned in a metal washer sealed by Permount (Fisher) to the center of a glass bottomed tissue culture dish (Delta T, Fisher). The depression was filled with BABB and a coverslip applied with Permount (Fisher).

Lysosome detection and autophagy

Both immunohistochemistry and live cell imaging were used to visualize the lysosomes and autophagic markers. Lysosome abundance and distribution in developing germ cells was determined using a rat antibody to Lamp1 (a lysosomal membrane glycoprotein; Developmental Studies Hybridoma Bank, University of Iowa, 1:100, cat. # 1D4B), and goat anti-rat Alexa Fluor 488 secondary antibody in both paraffin sections and whole mounts as described above. For analysis of acidic organelles in living tissues, intact ovaries [embryonic 19.5 and post–natal days 0 (day of birth), 1 and 2] were cultured for 4h and 18h in Acridine Orange (1µg/ml; Molecular Probes-Invitrogen) or LysoSensor Yellow/Blue DND (10µg/ml; Molecular Probes-Invitrogen). Individual ovaries were cultured in 100µl drops of culture media [MEM- with Earl Salts and L-Glutamine without phenol red (Gibco); 10% Fetal Calf Serum (FCS, Hyclone), 100ng/ml of Leukemia Inhibitory Factor (LIF, Sigma), 100ng/ml of Stem Cell Factor (SCF/c-Kit, PreProtech), 50ng/ml of Insulin-like Growth Factor I (IGF-I, Sigma), and 1% Gentomycin (Sigma)], covered with mineral oil (Sigma) and incubated with at 37°C with 5% CO_2. Glass bottom culture dishes allow live imaging of intact ovaries after 4 or 18 hours of culture and Hoechst 33342 was used as a vital nuclear stain. Lamp1 was quantified using Integrated Morphometry Analysis (IMA) software (MetaMorph version 7.5 Universal Image Corporation, USA), to

define individual germ cell boundaries (ROI, regions of interest) and measuring the area occupied by the Lamp1 signal. Values are represented as the percentage of Lamp1 per germ cell.

***In vitro* starvation of neonatal ovaries**

To create *in vitro* conditions resembling the nutrient-deprived state of neonatal ovaries, P0 and P1 ovaries (3 per group from different litters) were incubated in 600µl Complete Organ Culture Media [DMEM:Ham's F12, 1:1; 10% FBS, 1% ITS (Sigma), 1% Penicillin/Streptomycin (P/S; Sigma)], suspended in a 3µm PCF insert Millicell (Millipore), and placed in a 24 well tissue culture plate. Following a stabilization period, ovaries were either incubated for an additional 21h in Complete media or Starvation media (DMEM + 1% P/S). At the end of the total 24h incubation period, LTR (1%) was added to each well. For 0h control ovaries, LTR was added for 3h following the initial stabilization period. Hoechst 33342 was typically added as a nuclear counterstain. Ovaries were washed in PBS and fixed as for LTR in 4% PFA prior to confocal microscopy without BABB clearing.

Effect of apoptosis and autophagy inhibitors on starved neonatal ovaries

Using Complete or Starvation media (as above), E19.5 or P0 ovaries were incubated with inhibitors of programmed cell death. For apoptosis the pan-Caspase Inhibitor 1 ZVAD (OME)-FMK (50µM; Calbiochem) was used; for autophagy, 3-Methyladenine (10mM; Sigma) was used. Drugs were added alone or in combination for 21h and LTR and Hoechst 33342 were added for the final 3h of incubation. Tissues were analyzed by confocal microscopy to determine the volume density of LTR labelled germ cells in Z-section composites of whole ovaries using IMA software as above. Briefly, LTR positive objects with a diameter greater than 80µm^2 (determined to be the approximate diameter of germ cells) were thresholded and counted within 3-dimensional projections of a single ovary from each treatment group (n=3 per group). Approximately 50% of the entire ovary volume was

assayed for each replicate in each treatment group and the data are expressed as mean density volume per group.

Western blot analysis of apoptosis and autophagy proteins

Ovaries from foetal (E19.5), neonatal (P0, P1, P2, and P6), and pubertal (P20) mice were washed in cold PBS (4°C), and lysed [20mM Tris HCl (pH 7.4; Sigma), 150mM of sodium chloride (Sigma), 1mM of EDTA (Sigma), 1mM EGTA (Sigma), 1% Triton, 2.5mM sodium pyrophosphate (Sigma), 1mM β-glycerolphosphate, 1mM sodium orthovanadate (Sigma), 1µg/ml leupetin (Sigma), 1mM phenylmethanesulfonyl fluoride (PSMF; Sigma)]. Twenty micrograms of protein from pooled samples (E19.5, P2, 6, 16, and 20) was loaded on 12% sodium dodecyl sulfate–polyacrylamide (SDS-Page) gels and transferred to a polyvinylidene fluoride (PVDF) membrane (Millipore). Membranes were incubated for 1h in PBS-Tween 20 (0.05%) containing 5% nonfat milk and probed with primary antibodies to Caspase-3 (Cell Signalling cat. # 9661), LC-3B (Cell Signaling cat. # 2775), Cathepsin D (Santa Cruz, cat. # 10725) and Beclin 1 (Sigma cat. # B6061) followed by incubation with the appropriate horseradish peroxidase-conjugated secondary antibody (Southern Biotechnologies Associates); bands were detected with SuperSignal West Pico chemiluminescent substrate (Pierce) and an anti-actin antibody (Sigma cat. # A2066) was used to verify comparable loading between samples.

Image acquisition and analysis

Whole mount or sectioned ovary preparations were imaged using a LSM-510 Pascal confocal microscope (Zeiss, Germany) mounted on a Zeiss Axiovert 200M microscope equipped with excitation specific Diode (405nm), Argon (458, 477, 488 and 514nm) and Helium Neon lasers (594nm). Single scans or Z series data sets were made using 20x, 40x and 63x (NA=1.25) objectives and data was archived and analyzed using LSM software. Bright field images were obtained using a Nikon Eclipse 8i

microscope with 20x and 100x objectives and a Sony XWaveHAD colour camera.

Statistical Analysis

Data are presented as mean ± s.e.m. (Standard Error of the Mean) and statistical analysis of follicle number counts was performed using Prism 4.0 (GraphPad Software Inc., San Diego, CA). Data was analyzed by calculation of Confidence Intervals (CI) and non-parametric Kruskal-Wallis test of significance. It was considered statistically significant in cases where $P < 0.05$.

RESULTS

Characterization of germ cell number during ovarian development

Given the wide range of variability reported for germ cells during ovarian development in mice, we adopted a technical modification for our morphometric analyses. Here, histological sections processed for detection of mouse vasa homologue (MVH), a specific germ cell marker (Fujiwara *et al*., 1994) combined with periodic acid Schiff's (PAS) staining so as to render extracellular matrix, including the zona pellucida, clearly discernible (Fig. 1). Sections of ovaries from C57Bl/6 females ranging in age from embryonic day (E) 15.5 through to postnatal day (P) 150 were morphometrically analyzed for total oocyte, follicle, and/or zona pellucida profiles (Fig. 2 and Table SI). Examples of embryonic, postnatal, and prepubertal ovaries are shown in figure 1 to emphasize the striking histological transformation that takes place over these key developmental stages that include resolution of ovigerous cords into solitary follicles (Fig 1A-B) and expansion of somatic cells including development of the vasculature (Fig 1C).

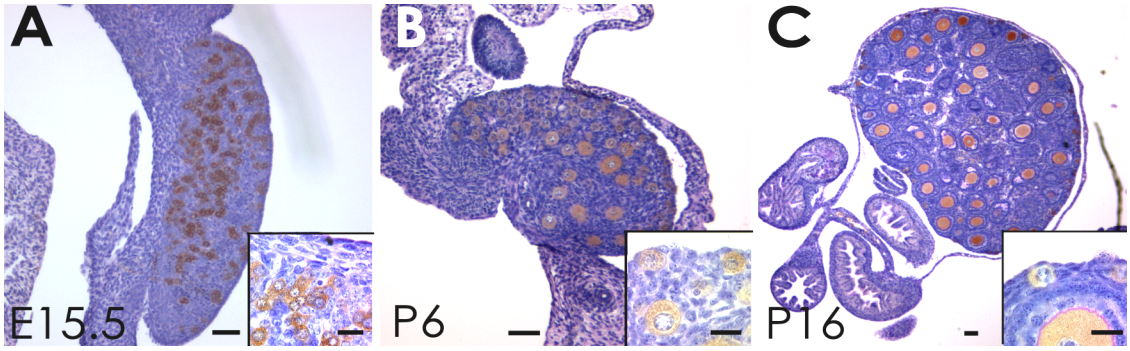

Figure 1. Germ cell disposition during ovarian histogenesis. MVH immunocytochemistry (brown reaction product) defines developmental variance in germ cell density and location. (**A**) Embryonic day 15.5 ovary, showing germ cell clusters and relative paucity of somatic tissue (inset). (**B**) Postnatal day 6 ovary, with cortical primordial follicles and medullary primary follicles; note apposition of peritoneal mesothelium to ovarian surface defining bursal cavity. (**C**) Postnatal day 16 ovary illustrating onset of antrum formation in centrally located follicles and cortically disposed preantral follicles with growing oocytes; insert shows 2 primordial follicles near surface and subtended by a primary follicle. Scale bars = 40µm for A, B and C, whereas for insets scale bars = 20µm.

In this study we confirm the decrease in mean germ cell numbers per ovary at birth, with 5783±475 (CI: 4463-7103) total follicles observed prior to birth (E19.5) and 3252±820 (CI: 976-5529) total follicles after birth (P2) (Fig. 2 and Table SI). Variations in mean oocyte number were also evident from immediately after birth through to the onset of reproductive cyclicity (data not shown). It should be emphasized that although germ cell number between P2 and P20 remains unchanged, considerable variability is observed between animals as expressed by the wide confidence intervals found for the mean numbers of total follicles at ages P2 (CI: 976-5529), 12 (CI: 264-4996) and 20 (CI: 947-5399) (Fig. 2). In sharp contrast to the lack of stability in germ cell number observed pre-pubertally, the rate of decline in follicle number from the onset of cyclicity (P42, CI: 1830-3713 total follicles) through adulthood is consistent between time points and animals. This was further confirmed by performing similar analysis on animals in which stages of the oestrous cycle were monitored (data not shown). Again, germ cell counts, *corpora lutea*, and atretic follicles, were similar at the age evaluated (P100, CI: 1419-2579 total follicles). Furthermore, estimates of the total number of zona pellucida remnants as a function of animal age also showed that their appearance coincided with the onset of reproductive

cyclicity, from undetectable at P20 to 20.8±3.2 at P42 and 34.9±1.7 at P100 (mean number per ovary for n=5 animals for each time point). These results are consistent with little if any net change in follicle number during prepubertal life and therefore prompted further enquiry into the nature of germ cell loss at perinatal developmental transitions.

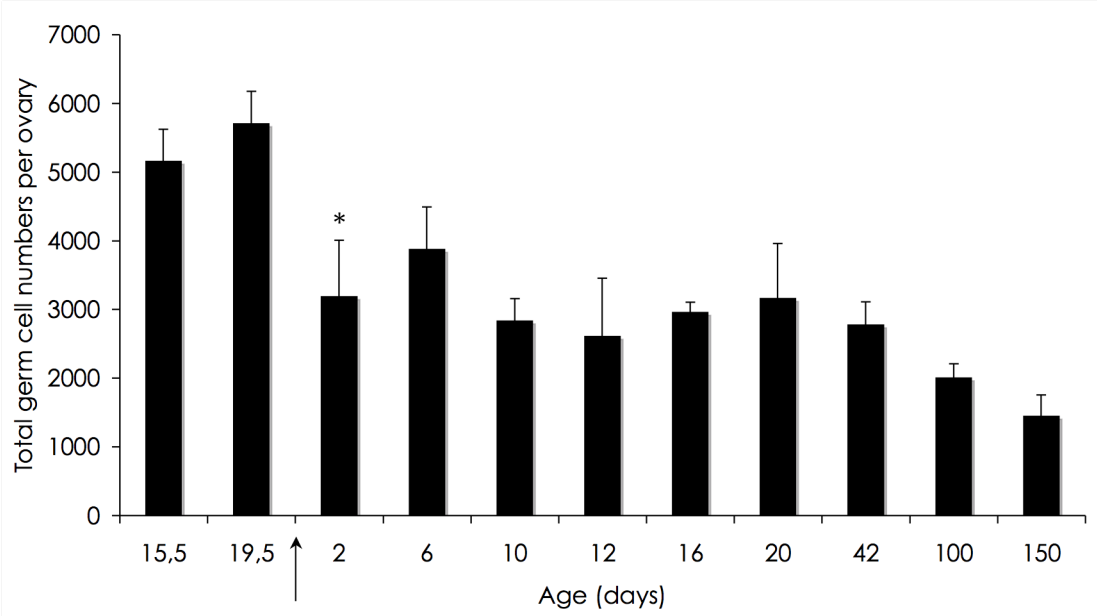

Figure 2. Mean number of total germ cell number per ovary from embryonic day 15 (E15.5) through to adult day 150 (P150). Note marked decrease in mean germ cell number following birth (P<0.05), and animal-to-animal variance in oocyte density between days P6 and P20 of age. Onset of cyclicity (P42) coincides with decrease in mean germ cell numbers that steadily declines up to P150. Data presented as mean ± s.e.m. (n = 5 animals per age group). Arrow indicates day of birth (day 20 post coitum).

Germ cell apoptosis during pre- and postnatal ovary remodelling

We first investigated the expression of various apoptosis markers prior to and following birth. Two common markers for early apoptosis, active caspase 3 and cleaved Poly (ADP ribose) Polymerase (Parp) were evaluated and the more traditional TUNEL assay for terminal stages of apoptosis. E15.5 and E19.5 samples exhibited limited staining for all markers in germ and somatic cells (Fig. 3A-C). This relative paucity of apoptotic germ cells was also evident in postnatal samples (Fig. 3D-F). As shown below, large clusters of germ cells were often seen at the surface of P2 ovaries, but even these clearly discarded oocytes were only rarely found to be positive for apoptotic markers. In contrast, ovaries from adult cycling

females exhibited the apoptosis patterns well established for mammalian ovary (Fig. 3G-I), with granulosa cells, but not oocytes in atretic follicles positive for apoptosis markers. The rarity of apoptotic germ cells prior to and following birth is difficult to reconcile with the 40% reduction seen by us and others and prompted investigation of alternative cell death mechanisms.

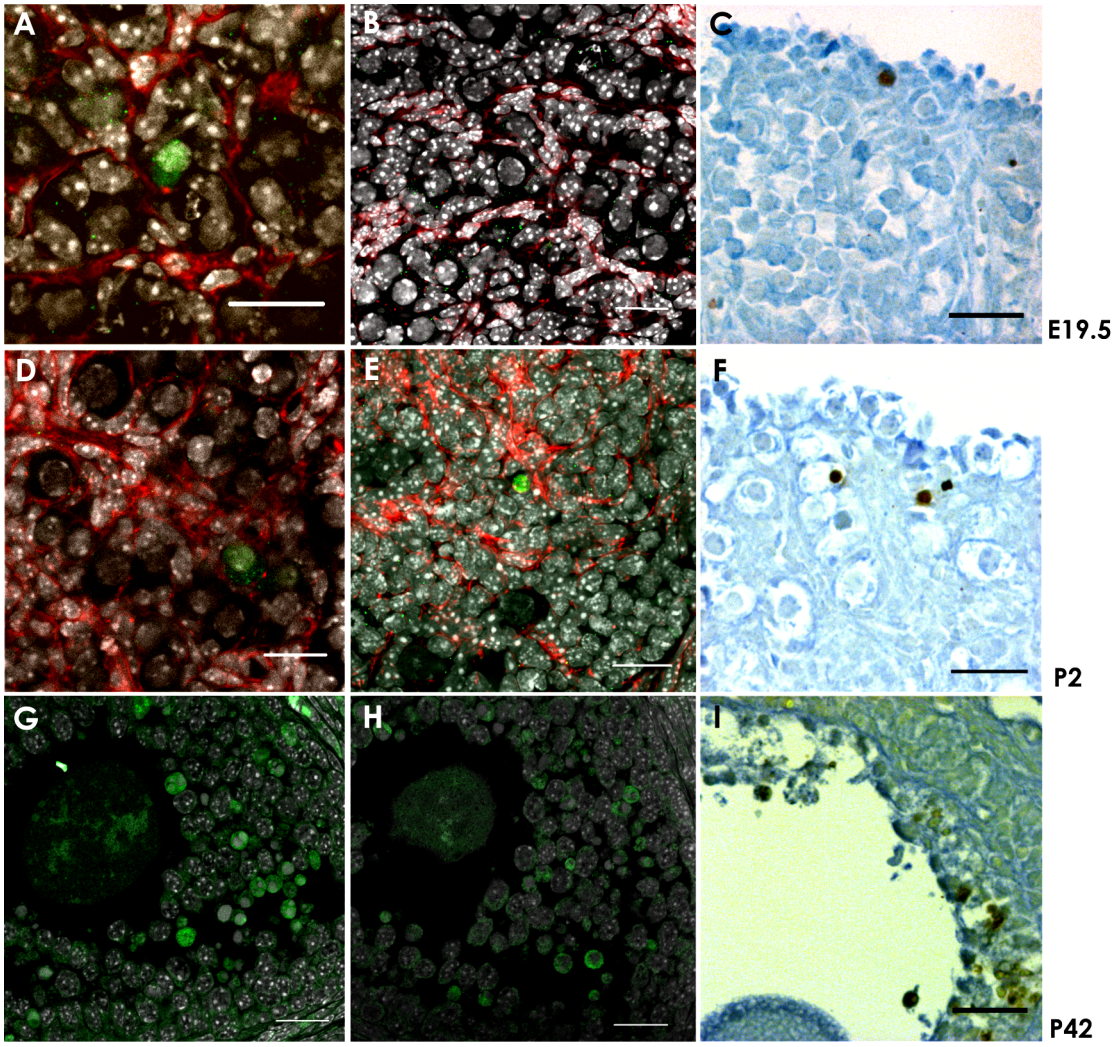

Figure 3. Pre- and postnatal apoptosis patterns. Comparison of apoptosis detection by cleaved-Parp (**A**, **D**, and **G**), active Caspase-3 (**B**, **E**, and **H**) or TUNEL (**C**, **F**, and **I**) assays in E19.5 (**A**, **B**, and **C**), P2 postpartum (**D**, **E**, and **F**), and P42 (**G**, **H**, and **I**) C57Bl/6 ovaries. (**A**) Confocal optical section of intact E19.5 ovary showing a solitary cleaved-Parp positive (green) germ cell amongst many negative germ cells (f-actin red, nuclei white). (**D**) Confocal optical section of intact ovary (P2) showing absence of staining in germ (one exception) or somatic cells for cleaved-Parp. Also note rare TUNEL positive cells in comparable ovaries (**B, E**). (**G**) (PARP red, GDF9 green), (**H**) (caspase red) and (**I**) (TUNEL brown) show that under identical processing conditions, apoptosis is readily demonstrable in granulosa cells of antral follicles in day P42 ovary. Scale bars = 20µm.

Postnatal ovarian histogenesis and germ cell extrusion

We first evaluated germ cell extrusion or shedding, as a mechanism of germ cell loss. Oogonia and primordial follicles were scored based on their position relative to the ovarian epithelium; these were classified as extra-ovarian if in the bursa cavity or intra-epithelial if positioned at the ovarian surface (Fig. 4A-D). As summarized in figure 4E, the mean number of extra-ovarian oogonia/oocytes observed in the bursa account for a minor fraction (~2%) of the total germ cells recorded at these time points. Intra-epithelial oogonia/oocytes represent a transient population of about 300 follicles at each stage examined up to P42, after which time they are not detected (Fig. 4E). Because of the difficulty in determining the flux rate of oocyte extrusion in fixed samples, we conducted live imaging studies in ovarian organ cultures. Intact E19.5, P0 (day of birth), and P1 ovaries were cultured for 4h or 18h. Germ cells associated with the ovarian surface were large and spherical and rarely contained associated somatic cells (Fig. S1) whereas those that accumulated on the bottom of the culture dish after 4h in culture were found in clusters of 4-8 cells (Fig. S1). While active extrusion was observed at ages examined, attempts to calculate a rate of loss were unsuccessful. An estimated extrusion rate of 150 germ cells per ovary over an 18h culture interval for P0 ovaries was obtained (Fig. S1) but in the absence of a way to follow single germ cells, we conclude that this mechanism of loss accounts for only a minor fraction of the loss observed until a more systematic analysis can be performed.

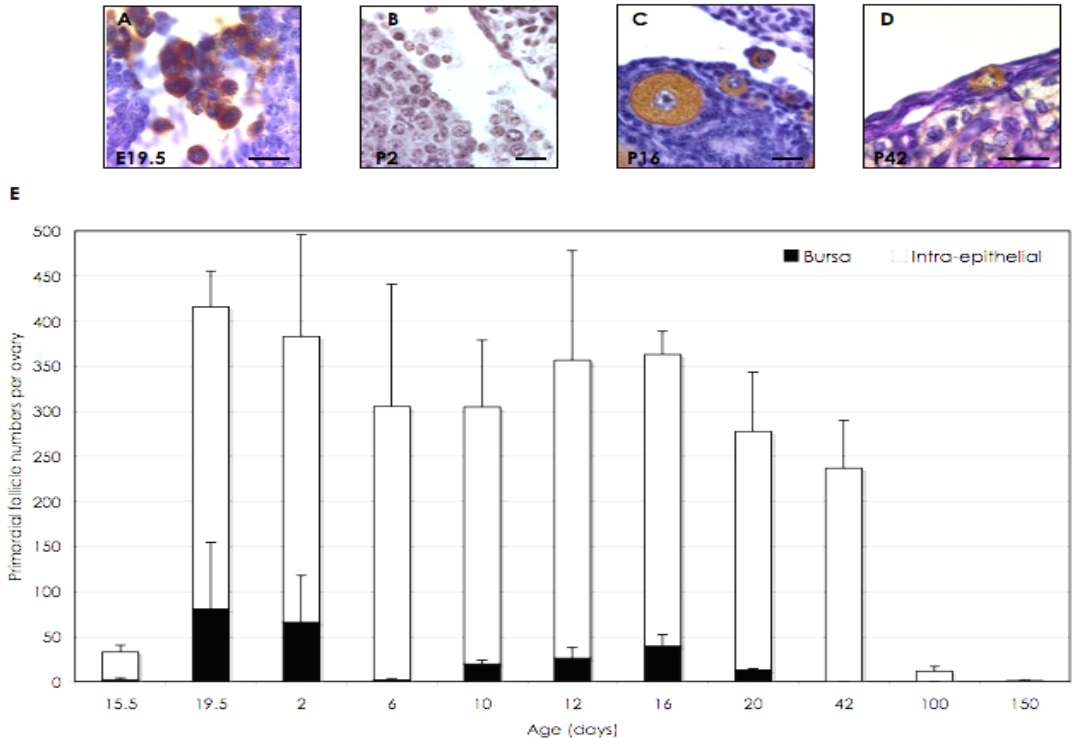

Figure 4. Developmental pattern of germ cell extrusion in mouse ovary. Germ cells were classified as extra-ovarian when within the bursal cavity (**A-C**) or intraepithelial when associated with or just beneath ovarian surface (**D**). (**A-D**) Bright field images of sections stained with MVH (brown), hematoxylin (blue) and PAS (pink). Bars = 20 µm. (**E**) Comparison of extra-ovarian (filled bars) or intraepithelial (open bars) germ cells over developmental age range studied (E15.5-P150). Mean number primordial follicles ± s.e.m. per time point are plotted (n= 5 ovaries/age).

Lysosome compartment amplification and autophagy

Since lysosomes are involved in both autophagy and apoptosis (Guicciardi *et al*., 2004), we first analyzed their expression in somatic and germ cells of perinatal ovaries. Using Lamp1 antibody, which detects a membrane constituent of functional lysosomes, a striking increase in lysosome density was observed from E19.5 to post-natal ovaries (Fig. 5A-B). Figures 5C and D show further that the increase in Lamp1 staining in germ cells coincides with the onset of oocyte growth and follicle assembly. Quantification of Lamp1 by image analysis of stained ovarian sections further confirmed the lysosome amplification post-birth (Fig 5E). Significant changes in the amplification of lysosomes were observed from E19.5 2,02±0.21 (CI: 1.60-2.43) to P2 15,08±0.95 (CI: 13.19-16.19) by measuring the mean Lamp1 staining per germ cell. The general pattern of increased lysosome expression after birth was also seen with the vital lysosome marker acridine

orange (data not shown). Thus lysosome amplification in all oocytes occurs upon birth and as in other postnatal tissues may represent an adaptive response to postnatal starvation. To directly address this question, an additional experimental strategy was used to ascertain the relative contributions of apoptosis and autophagy in the perinatal mouse ovary.

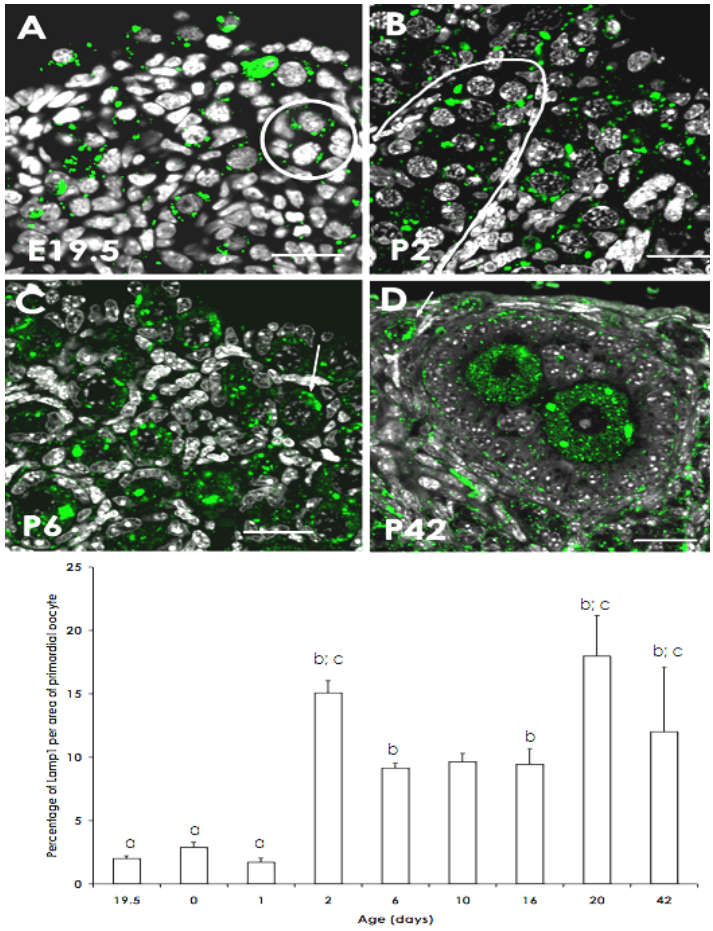

Figure 5. Lamp 1 expression in neonatal mouse ovaries. Prenatal ovaries (**A** and **B**) exhibit Lamp1 stained lysosomes (green) predominantly in germ cells (somatic and germ cell nuclei are white). After birth, large lysosomes are evident in oocytes that form primordial follicles (**C-D**, arrows) whereas smaller lysosomes within oocytes that are not fully enclosed by follicle cells (**B**, arrowheads). (**E**) Shows quantitation of Lamp staining in oocytes between E19.5 and P42; note the abrupt increase at P2 and stabilization of lysosome density from P16 on. Columns with different letters are significantly different from each other ($P<0.05$). Scale bars = 20µm.

We adapted the methods of Zucker and colleagues (1998) to image intact ovaries that had been labelled with the probe LysoTracker Red (LTR) using high-resolution confocal microscopy to monitor cytoplasmic and organellar acidification in tissues in an unperturbed state. In E19.5 ovaries,

LTR reveals smaller apoptotic cells, macrophages identified by multiple lysosomes, and larger germ cells evidenced by acidified cytoplasm (Fig. 6A-B). Acidified LTR labelled germ cells were common at P2, but rare at later stages of ovarian development (Fig. 6C-D). We next tested the effect of serum starvation on the loss of germ cells in an ovarian organ culture system.

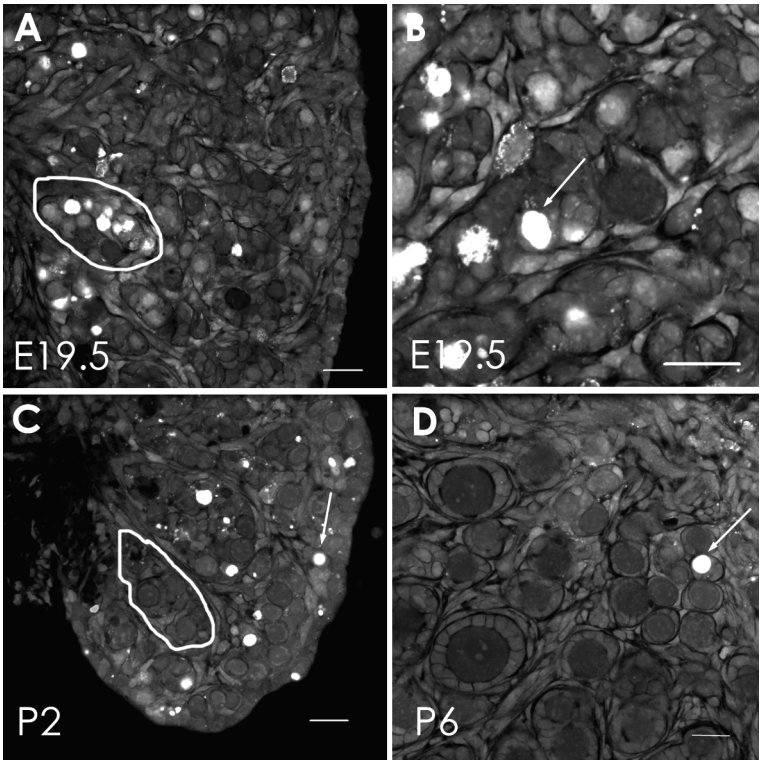

Figure 6. Analysis of LTR staining in intact mouse ovaries. E19.5 ovaries (**A** and **B**) just prior to birth containing both cytoplasmic acidification (arrows) and LTR foci are seen within germ cell cluster (some circled for easy visualization). From P2 (**C**) to P6 (**D**), seem to have fewer cells with cytoplasmic acidification (arrows). Scale bars = 20µm.

To accomplish this, ovaries from P0 animals were cultured and exposed to LTR in the presence or absence of serum for 24h. Complete Z stack projections were thresholded to demarcate acidified germ cells (Fig. 7A-C and Fig. S2), which were counted and expressed as mean density volume per ovary (7G). Direct comparison between P0 (56.93±8.86; CI: 18.80-95.08) and P1 (mean of 20.99) for ovaries showed a 60% decrease at these time points with freshly isolated tissues. Comparison of serum treated (14.40±3.64; CI: -15.41-44.22) versus serum starved (23.61±5.08; CI: 1.77-45.45) organ

cultures illustrates a higher proportion of acidified germ cells after serum removal (Fig. 7G). These findings indicate that maintaining newborn ovaries in the presence of serum lessens the incidence of autophagy.

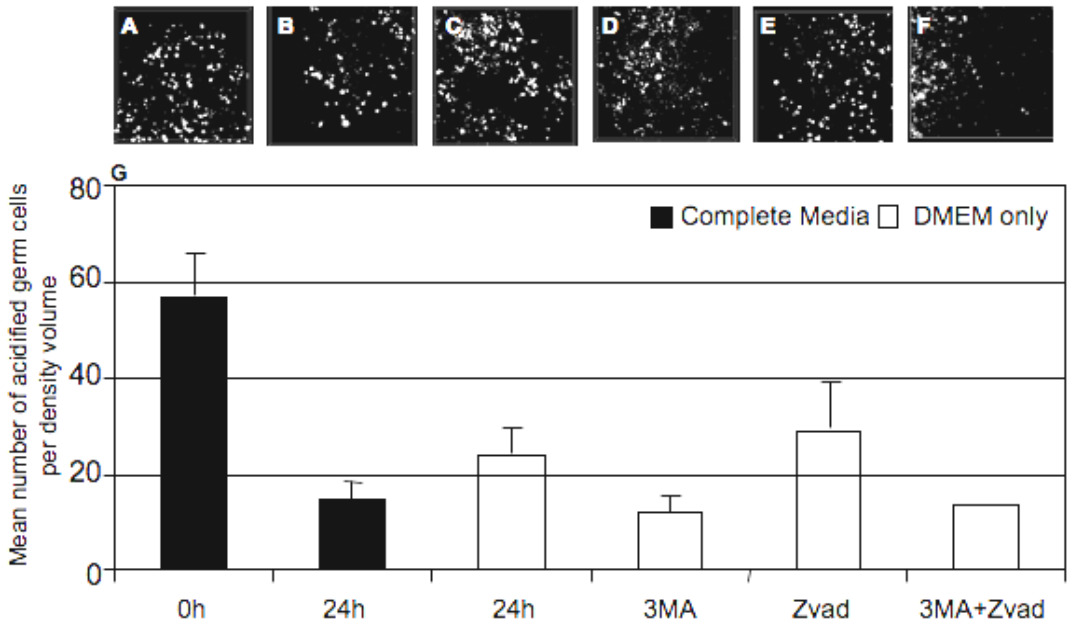

Figure 7. Quantitation of acidified germ cell density volume (G) in perinatal mouse ovaries cultured with (filled bars) and without (open bars) serum in the presence or absence of autophagy (3MA) and/or apoptosis (ZVAD) inhibitors (open bars). Panels at top represent complete confocal projections of intact ovaries after image thresholding to reveal germ cell density for each treatment group. Note decreased prevalence of LTR positive cells when ovaries are maintained in complete medium for 0h (**A**) and 24h (**B**) compared to serum deprivation for 24h in culture (**C**). The autophagy inhibitor 3MA, either alone (**D**) or in combination with ZVAD (**F**) reduces the density of acidified germ cells under serum free conditions. Note that treatment with ZVAD alone (**E**) has little effect on germ cell density compared to controls (**C**). (**G**) Values presented as mean±sem (n=3 ovaries except for combination of the 2 inhibitors, for which n=2 ovaries).

We next tested the effects of apoptosis or autophagy inhibitors on the density of LTR positive germ cells.

Autophagy as an effector of germ cell loss in the newborn ovary

3-methyl adenine (3MA), a commonly used autophagy inhibitor (Boya *et al.*, 2005; Seglen and Gordon, 1982), and ZVAD, a commonly used pan-caspase inhibitor (Boya *et al.*, 2005; Kim *et al.*, 2001) were used to selectively impair autophagy or apoptosis in the organ culture system described above. Ovaries (n=3) were exposed to either inhibitor alone or a

combination of both inhibitors for 24h and processed for LTR staining. In the presence of 3MA, an apparent reduction in the density of acidified cells was evident (7D). In contrast, ZVAD treatment had little discernible effect on the density of acidified cells compared to control ovaries (Fig. 7E and C, respectively and Fig. S2). Interestingly, exposure to both 3MA and ZVAD (Fig. 7F) for 24h reduced LTR-positive cell density to levels comparable to those observed in the 3MA treatment group (Fig. 7D and Fig. S2). These trends were confirmed by quantitative image analysis (Fig. 7G). Whereas the density of LTR-positive cells was similar in control (24h, 23.61±5.08; CI: 1.77-45.45) and ZVAD (28.77±8.86; CI: -9.36-66.90) groups, 3MA alone (12.05±3.45; CI: -2.79-26.90) or in combination with ZVAD (13.38±1.28; CI: -2.89-29.85) resulted in cell densities comparable to those attained after culture in the presence of serum (14.41±3.64; CI: -15.41-44.22). Collectively, these results indicate that inhibition of autophagy and not apoptosis influence germ cell loss in newborn ovaries. This was further supported by an analysis of protein expression in perinatal ovaries (Fig. 8). Mediators of apoptosis (caspase-3, cathepsin D) and autophagy (LC-3B and Beclin 1) were probed by western blots of pooled ovary samples from animals of E19.5, P2, P6 and P20 ages.

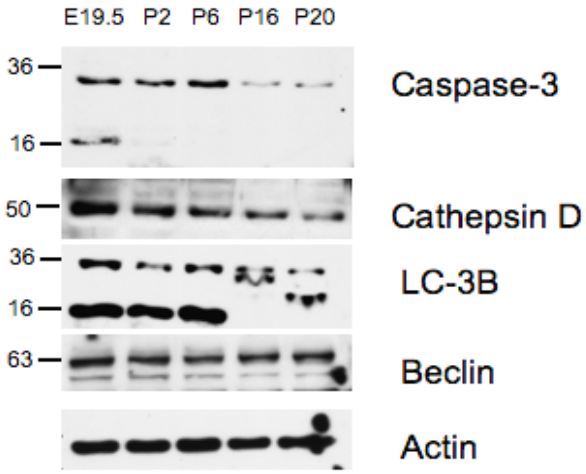

Figure 8. Apoptotic and autophagic protein expression. Western blot analysis of caspase-3 and Cathepsin D (apoptosis), LC-3B and Beclin 1 (autophagy), and β-actin expression in E19.5, P2, P6 and P20 pooled ovaries.

Figure 8 shows active caspase-3 protein expression on E19.5 (~17KDa) alone, and inactive caspase-3 expression (32KDa) through all ages examined. Notably, active caspase-3 is only detected in E19.5 day samples. Expression of the 16KDa LC-3B, the autophagosomal membrane form of LC3B (Tanida *et al*., 2005), was evident between E19.5 and P6. The 36KDa form of LC3B decreases at birth and remains relatively stable in expression at the later time points. Interestingly, cathepsin D gradually decreases until P20. Beclin levels show a slight decline at birth but also remain stable through to P20. Collectively, these data indicate that mediators of apoptosis and autophagy are differentially expressed while pre- and postnatal germ cell loss takes place.

DISCUSSION

Most female eutherian mammals establish a stockpile of follicle-enclosed oocytes at or soon after birth that is gradually depleted through the organisms' reproductive lifespan. In such species, it has long been appreciated that the most profound period of germ cell loss occurs shortly after parturition (Faddy and Gosden, 2007). Moreover, it is generally held that the primary determinant of germ cell survival involves parallel processes of germ cell enclosure in the primordial follicle and selective susceptibility of some oocytes to type I PCD, apoptosis (Coucouvanis *et al*., 1993; De Pol *et al*., 1997; Pepling and Spradling, 2001; Reynaud and Driancourt, 2000; Tilly, 2001). Since oocyte attrition is a fundamental tenet of reproductive aging and certain fertility disorders in women, there has been general interest in the mechanisms responsible for both the establishment and maintenance of the female germ cell supply (Gosden, 2002; Matzuk, 2005; Skinner, 2005). Here, we used the commonly studied C57Bl/6 mouse strain to gain insight into the mechanisms of perinatal germ cell loss. While our results concur with previous studies indicating that substantial germ cell attrition occurs at the onset of reproductive maturity and birth, they also question type I PCD as the sole cause for early germ cell loss. Instead,

multiple perinatal mechanisms (apoptosis, germ cell extrusion, autophagy) interact to establish a finite follicle reserve for later use. The conclusion that different PCD mechanisms operate at discrete stages of ovarian development suggests a level of complexity in the regulation of ovarian germ cell and follicle survival that has only recently been appreciated (Lobascio *et al*., 2007).

Ovarian developmental transitions engage distinct PCD mechanisms

Unlike earlier histomorphometric analysis of germ cells and follicles in the mouse ovary, our methods using immunostaining of MVH for unequivocal germ cell identification provided an accurate counting method for analysis of all follicle stages from E15.5 to P150 (Fig. 2). This kind of analysis confirmed the sharp decline in oocyte number at birth noted by others (Baker, 1966; Coucouvanis *et al*., 1993; Kerr et al., 2006; McClellan *et al*., 2003; Ratts *et al*., 1995) and further showed that over the postnatal period approaching puberty (P6-P42), little change in follicle numbers was detected. Our results are in agreement with those of Kerr et al., (2006) (Table SI). Many studies have demonstrated the occurrence of germ cell loss through apoptosis in foetal mouse ovary (Coucouvanis *et al*., 1993; De Felici *et al*., 2005; Pepling and Spradling, 2001) and in some cases have linked type I PCD to errors in meiotic cell cycle checkpoint control (Burgoyne and Baker, 1985). It should be emphasized, however, that the frequency of TUNEL-positive oocytes seen in foetal mouse ovary is surprisingly small ranging from 0.5 to 5% (Pepling and Spradling, 2001; Pesce *et al*, 1997). A recent re-examination of this stage of ovarian development by Lobascio *et al*., (2007) has concluded that previous studies failed to recognize the complexity of germ cell loss at foetal stages. Interestingly, these authors called attention to the fact that the mTOR inhibitor, rapamycin, increased TUNEL staining in germ cells presumably due to the negative effect of mTOR on autophagy. Moreover, calpain inhibitor 1 also induced apoptosis in germ cells after prolonged culture as evidenced by

the appearance of caspase-negative and atypical TUNEL-positive oocytes (De Felici *et al*., 2007; Lobascio *et al*., 2007). The rare occurrence of apoptotic cells in perinatal ovaries described here further demonstrates that additional, programmed cell death mechanisms, such as autophagy, are involved with germ cell attrition prior to and after birth in the mouse. These findings are immediately relevant to strategies seeking to prevent germ cell loss due to therapeutic or genetic causes and emphasize the need to clearly identify the PCD mechanisms involved.

Germ cell extrusion during ovarian morphogenesis

Oocytes have been noted to reside close to or within the ovarian epithelium in previous studies (Albertini and Barrett, 2004; Hirshfield, 1992; Motta *et al*., 2003; Wordinger *et al*., 1990) but the relative rarity of these and their disappearance with further postnatal development of the ovary have led most to conclude that this is a minor cause of germ cell loss (Byskov, 1982; Hirshfield, 1992; Motta *et al*., 2003). Estimates based on the present studies would tend to support this idea based upon analysis of static images at various time points. While this process could result in loss of a significant fraction of oocytes resident at the time of birth, a definitive assessment awaits development of technologies suitable for an analysis of this dynamic process.

Autophagy: an alternative mechanism for perinatal germ cell loss

The perinatal period of life in placental mammals marks an abrupt transition in energy homeostasis. Mounting evidence shows that up regulation of autophagy follows in the immediate hours of postnatal life in many tissues and organs (Kuma *et al*., 2004). It is now accepted that this burst of autophagy is an adaptive response to nutritional stress once the newborn organism is deprived of placental nutrients (Kuma *et al*., 2004). Coupled with studies on foetal ovaries cited above (De Felici *et al*., 2007; Lobascio *et al*., 2007), our results implicate autophagy in the establishment

of the follicle reserve. That multiple PCD mechanisms would be involved in this process is not surprising since it has been shown in many other systems (Kuma *et al*., 2004). Moreover, ultrastructural studies of mammalian oocytes have repeatedly documented the presence of lysosomes and autophagosomes in many species (Hertig and Adams, 1967; Wassarman and Albertini, 1994) but the developmental time course of their appearance and its relationship to autophagy have not been established.

Lysosome amplification is a hallmark of autophagy (Bursch, 2001). Using Lamp1 immunocytochemistry and vital markers for lysosomes, we document amplification of lysosomes in oocytes upon birth (Fig 5). Moreover, accumulation of lysosomes was most apparent in oocytes enclosed within primordial follicles whereas these structures were less apparent in oocytes that were either extruded through the ovarian surface or undergoing PCD.

However, discriminating between germ cells actively undergoing autophagy or apoptosis required application of a vital staining method that would permit assessment of germ cell behaviour at critical developmental stages. For this, we used the LTR method and confocal microscopy so that intact living ovaries could be evaluated by digital image analysis (Fig. 6). Foetal and neonatal ovaries exhibited cytoplasmic acidification of germ cells that were characterized by their size and disposition. These germ cells were distinct from resident macrophages and smaller germ cells that exhibited fibrillar staining after LTR labelling. Quantitation of germ cells at (P0) and just after birth (P1) indicated a gradual decline in their density at this transition. Importantly, ovaries from newborn animals cultured in the presence of serum-enriched medium displayed fewer LTR positive germ cells than those maintained in a serum-free environment This finding suggests that the removal of ovary from a growth factor-rich environment, rather than an endogenous signal, was

able to elicit germ cell death at the time when major loss is occurring. Growth factors are known to prevent follicle loss in both *in vivo* and *in vitro* models of ovarian development (Bristol-Gould *et al*., 2006; De Felici, 2000; Nilsson and Skinner, 2001; Skinner, 2005) and one likely candidate for maintaining germ cells is Kit Ligand (KL) (De Felici, 2000; Hutt *et al*., 2006; Parrott and Skinner, 1999). While this experimental approach would be helpful in defining the physiological factors that regulate follicle formation and survival under dynamic conditions, some studies involving section analysis have supported the general notion that growth factor deprivation at birth is responsible for much of the germ loss seen at this time (Bristol-Gould *et al*., 2006; Durlinger *et al*., 1999). Whether the effects of serum on germ cell loss were mediated by apoptosis or autophagy was next investigated using selective inhibitors for each of these processes.

As shown above (Fig. 7 and S2), the selective autophagy inhibitor 3MA (Boya *et al*., 2005; Seglen and Gordon, 1982) reduced the acidified germ cell density compared to controls whereas no effect was seen after treatment with ZVAD, a pan-caspase inhibitor. Moreover, use of both inhibitors reduced the density of acidified germ cells to the same extent as 3MA alone, suggesting further that autophagy rather than apoptosis is involved in germ cell loss after birth. That combinatorial mechanisms may mediate this process is further indicated by western blot analysis of components for either apoptosis or autophagy (Fig. 8).

Prominent perinatal expression of Beclin 1 and LC-3B (16KDa) in ovaries at E19.5, P2 and P6 directly implicate autophagy, but as also shown, activated caspase-3 (~17KDa) is present only in foetal ovaries. Since cathepsin D is also present over this time interval, it is tempting to suggest that apoptosis at low levels may occur both prior to and following birth whereas the activation of autophagy appears to be directly linked to parturition-induced starvation. Caspase-3 antibody also detects a 36KDa component that most likely is the inactive, constitutively expressed form

that requires cleavage for protease activation (Boone and Tsang, 1998). Moreover, 16KDa band corresponding to the LC3B II isoform is localized to autophagosomes and autolysosomes (Tanida *et al*., 2005). Together, these results support the existence of combinatorial mechanisms for germ cell loss and survival at birth. Further resolution of the interactions between both of these PCD pathways will be facilitated by the use of conditional knockouts of genes involved in each of these processes.

In summary, these results suggest that multiple PCD mechanisms function in the perinatal mouse ovary to establish the primordial follicle reserve. The relative contributions of apoptosis and autophagy may vary at distinct stages of ovarian development. The present findings will be of relevance to emergent technologies seeking to optimize and preserve high quality oocytes for experimental or clinical use. Furthermore, they uncover a level of complexity in female germ line development in eutherian mammals not previously appreciated. Future studies seeking to rescue female germ cells in newborns, adolescents or adults will need to take into account the diverse mechanisms that are at play during different stages in the lifespan of the mammalian ovary.

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SUPPLEMENTAL FIGURES AND TABLE

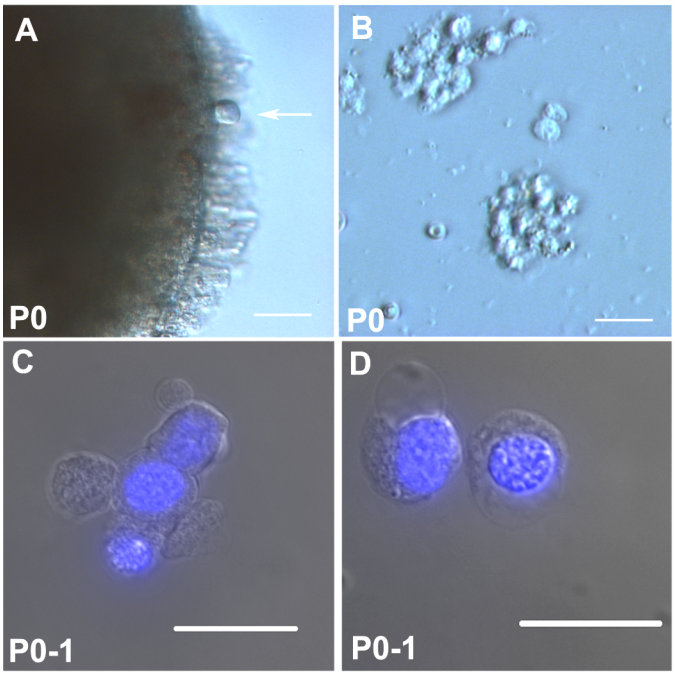

Figure S1. Active extrusion of germ cells in cultured perinatal mouse ovaries. (**A**) Margin of a P0 ovary cultured for 4h; note germ cells associated with ovarian surface (arrow). (**B**) Same ovary as in (**A**) shed germ cell clusters from ovarian surface after culture for 4h. (**C** and **D**) Extruded germ cells show diffuse chromatin after vital staining with Hoechst 33342; note blebbing from some cells. Scale bars = 20µm.

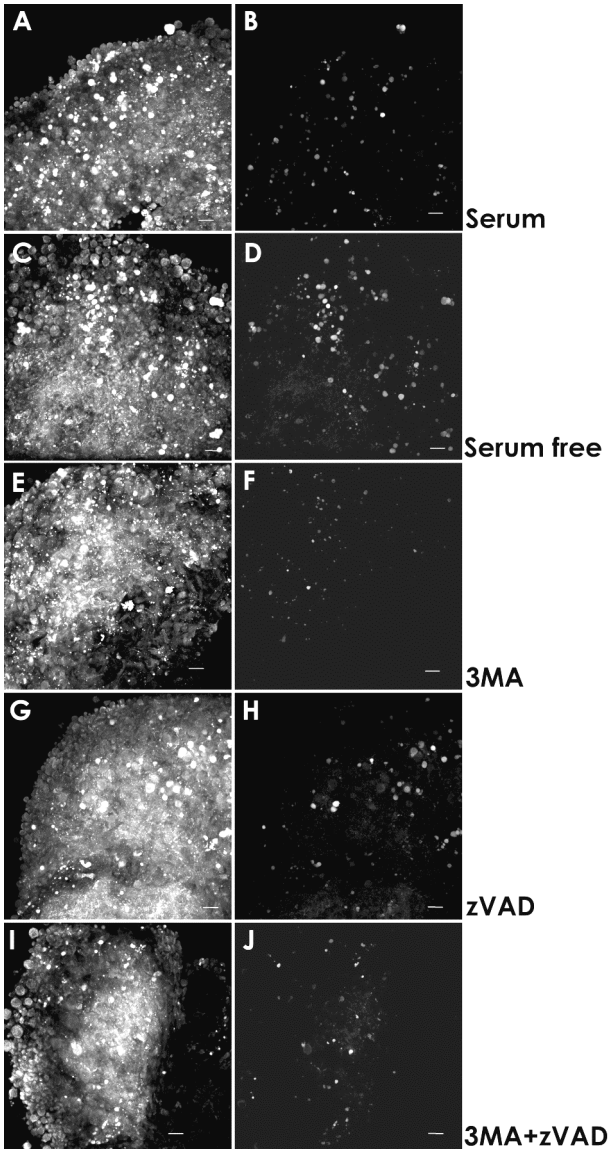

Figure S2. LTR labelling profile for mouse ovary organ culture. Panel on the left represent complete confocal projections of intact ovaries and on the right are the same ovaries after thresholding data sets to reveal LTR in cell with acidic cytoplasm under each organ culture treatment. (**A** – **D**) Perinatal mouse ovaries cultured for 24h in complete medium (with serum) (**A** - **B**) and serum depravation (**C** – **D**). Note that LTR positive cells increase when serum is absent from the organ culture media. (**E** – **J**) Perinatal ovaries cultured in serum free media in the presence of autophagy (3MA) and/or apoptosis (ZVAD) inhibitors. Autophagy inhibitor 3MA, either alone (**E** and **F**) or in combination with ZVAD (**I** and **J**) reduces the density of cells cytoplasm acidification after 24h culture under serum free conditions. Note that treatment with ZVAD alone (**G** and **H**) has little effect on the density of LTR positive cells compared to controls (**C** - **D**). Scale bars = 20µm.

Table SI – Total mean germ cell number as well as extra-ovarian germ cells distinguishing between: bursa and intra-epithelial. Total oogonia and/or primordial, primary, secondary, early antral and antral follicles, corpus luteum (CL) throughout development (n = 5 per age, data represented in mean ± s.e.m.). The confidence intervals (CI) for total follicle numbers are given in the last column.

Age	Total germ cells	Germ cells in specific locations		Total Oogonia or Primordial Follicles	Total Primary Follicles	Total Secondary Follicles	Total Early Antral Follicles	Total Antral Follicles	CL	CI for total follicles
		Bursa	Intra-Epithelial							
E15.5	5158±468	2±2	30±8	5156±469	0	0	0	0	-	3855-6457
E19.5	5783±475	80±74	335±40	5703±500	0	0	0	0	-	4463-7103
P2	3252±820	66±52	317±113	3182±822	5±3	0	0	0	-	976-5529
P6	3875±622	2±1	303±60	3615±607	256±19	1±1	0	0	-	2149-5601
P10	2848±332	20±4	285±74	2385±275	361±91	77±27	5±5	0	-	1927-3770
P12	2630±852	26±12	330±122	1928±658	216±56	108±11	10±10	0	-	264-4996
P16	2997±148	39±13	324±26	2486±149	244±39	201±25	27±16	0	-	2587-3407
P20	3173±802	13±2	265±66	2615±758	232±48	284±14	29±10	1±1	-	947-5399
P42	2772±339	0	237±33	2452±290	236±43	74±10	8±2	3±1	2±0.5	1830-3713
P100	1999±234	0	12±6	1612±232	285±20	83±10	14±2	5±2	6±0.4	1419-2579
P150	1444±314	0	1±1	962±277	249±18	154±18	65±11	14±3	6±0.5	572-2317

CHAPTER # 3

Perinatal ovarian remodeling and establishment of the definitive follicle reserve: the somatic-germ cell interface

(Patricia Rodrigues, Darlene Limback, Lynda McGinnis, David F. Albertini and Carlos E. Plancha – manuscript in preparation for submission)

ABSTRACT

Purpose: To define properties of germ-somatic cell interactions during the pre- to post-natal development in the mouse ovary.

Methods: Using cytoskeleton, extracellular matrix (ECM), adhesive and gap junction markers, we performed immunofluorescence in whole-mount and/or paraffin sections of mouse ovaries at the time of follicle assembly (foetal day 19.5 and postnatal days 2, 6 and 20).

Results: We found that germ-somatic-matrix cell interplay is fundamental to ovarian differentiation, including ovarian surface epithelium. ECM and junctional markers showed that the ovarian epithelium has not fully formed by postnatally day (P2) thus facilitating germ cell loss at early stages, and contributing to the establishment of the ovarian reserve. Analysis of cytokeratin marker expression demonstrastes full epithelial differentiation commencing on postnatal day 20. Connexin expression patterns indicate that gap junctions are present in the somatic cells (43; GJA1) and in the oolema (37; GJA4) throughout the timeframe we have investigated, pointing to their continuing involvement in ovarian ontogeny and follicle assembly. These observations are accompanied by Notch, distributed around germ-somatic interface, thus implying the presence of the Notch signaling pathway in this process, too.

Conclusions: establishment of ovarian follicle reserve requires coordinated differentiation of epithelial and stromal components that depend upon multiple steps of ovarian histogenesis and germ-somatic-matrix cell interactions during the final stage of follicle assembly.

INTRODUCTION

Building an ovarian follicle involves a complex interaction between various cell types. Traditional views in the mouse model hold that oogonia proliferate within a cluster, through a series of incomplete mitosis (Pepling and Spradling, 1998). These clusters are surrounded by mesonephros-derived somatic cells forming the ovigerous or rete cords, continuous with the surface epithelium of the ovary (Byskov, 1986; McLaren, 2003; Mazaud *et al*., 2005; van den Hurk and Zhao, 2005; Guigon and Magre, 2006). Within the ovigerous cords, at around 15.5 dpc, oogonia enter into prophase of the first meiotic division and will arrest in diplotene stage until stimulated to resume meiosis, shortly before ovulation (van den Hurk and Zhao, 2005; Jones and Pepling, 2013). Follicular histogenesis is initiated through a process that encompasses fragmentation of the ovigerous cords, alignment of epithelial pre-granulosa cells, and the attraction of mesenchymal cells to the follicle's nascent basement membrane (van den Hurk and Zhao, 2005; Guigon and Magre, 2006). This coordinated interaction results in the formation of the pool of primordial follicles, known as the ovarian follicular reserve.

Recently, mechanisms besides cyst breakdown were suggested, in mouse and bovine, for follicle assembly (Mork *et al*., 2012; Hummitzsch *et al*.,2013). In the mouse it was suggested that granulosa cells descend directly precursors cells of the gonad (Mork *et al*., 2012). In the bovine, it was proposed that ovarian surface epithelial cells penetrate into the ovary to form the granulosa cells, therefore these two cell types have a common cell ancestor, the GREL – Gonadal Ridge Epithelial-Like cells (Hummitzsch *et al*., 2013).

Accordingly, we have traced the origin and lineage of follicles in the developing perinatal mouse ovary, with special interest on somatic-germ cell and somatic-extracellular matrix interactions. We further focused on direct cell-cell interactions via gap junctions between the oocyte and surrounding granulosa cells because these figure prominently in nearly all aspects of folliculogenesis (Simon *et al*., 1997 (cx 37), Juneja *et al*.,1999; Ackert *et al*., 2001(cx 43); Albertini *et al*., 2001; Kidder and Mhawi, 2002; Motta *et al*., 2003; Simon *et al*., 2006). Here we report that ovarian reserve is derived from distinct origins for follicles that are developmentally separated in time and by the nature of the cell/cell matrix interactions.

MATERIALS AND METHODS

Animals

Inbred C57Bl/6 timed-pregnant mice (Charles River, Willington, MA) were used for all experiments and housed in a 14 hours light: 10 hours dark environment at constant temperature. Food and water was provided *ad libitum*. Mice were maintained and used in accordance with the policies of the University of Kansas institutional Animal Care and Use Committee (IACUC). Pregnant females were received at least two days prior to use to minimize the effect of shipping stress and allow adjustment to the new environment.

Ovary collection and tissue preparation

Females were euthanized by cervical dislocation. After euthanasia, ovaries were dissected from female fetuses at fetal day 19.5. Some pregnant females were allowed to deliver, which occurred on the early morning of day 20-post coitum. Female neonates were euthanized at days 2, 6, and 20. At least three ovaries per age were collected; the fetal ovaries were collected from at least 2 different pregnant females and the neonates from at least 2 different litters. Right and left ovaries from each animal were either fixed in Bouin's fluid (Sigma, St. Louis, USA) or 2% paraformaldehyde

(PFA, Sigma), for 4-6 hours at room temperature and overnight at 4ºC, respectively. Following fixation, ovaries were transferred to 70% ethanol and processed for paraffin embedding (processor Shandon, GMI, St Paul, USA).

Whole ovaries for confocal imaging were fixed in Microtubule Stabilization Buffer Extraction Fix [MTSB-XF: Stabilization Buffer: (stock 5x: Pipes (Sigma) 0,1 M, $MgCl_2.6H_2O$ (Fisher, USA) 5 mM and EGTA (Sigma) 2,5 mM); Aproptinin (Sigma); DTT (Sigma); D_2O (Sigma); Taxol (received from NIH); 1% Triton (stock 10%, Sigma); Formaldehyde (Fisher)], overnight at 4ºC and then stored in wash/block solution [PBS azide (with 0,2% azide, Sigma); 0,2% Powdered Milk (grocery); 2% Normal Goat Serum (NGS, Gibco – Invitrogen, USA); 1% BSA (Sigma); 0,1 M Glycine (Research Organics); 0,1% Triton X-100 (Sigma)] at 4ºC, for at least 24 hours.

Immunofluorescence

Paraffin sections

Slides were dewaxed and re-hydrated using conventional methods. Antigen retrieval was preformed, to those antibodies that needed it (see Table I), in a microwave for 15 minutes in citrate buffer (0.01M). Slides were gradually cooled to room temperature and blocked for 30 minutes in 9% goat serum (Zymed, USA) containing 3% Bovine Serum Albumin (BSA, Sigma). Sections were then incubated in primary antibody (see Table I) overnight at 4ºC, in a humid chamber. Following washes (3x, 5 minutes), sections were incubated in the respective secondary antibody for 1h at 37ºC. After washes (3x, 5 minutes), sections were incubated in ethidium-homodimer 2 (Eth-D2, 1:100; Molecular Probes, Invitrogen, USA), a nuclear dye, for 10 minutes at room temperature. Following two final washes, slides were mounted in Prolong (Molecular Probes, Invitrogen, USA). Control slides were processed identically but primary antibody was omitted.

All antibodies were diluted in Automation buffer BiØmedia, USA) with 1% BSA, and automation buffer was used for all wash steps.

Whole ovary

Fixed ovaries stored in wash/block solution were halved and either the left or right half was incubated in primary antibody (see Table I) with constant agitation, for 24 hours at 4ºC. All antibodies were diluted in wash solution. Tissues were subsequently washed (4x 1 hour; 37ºC) and placed in secondary antibody (see Table I) overnight at 4ºC with constant agitation. On the following day, ovaries were washed as before, with the addition of the nuclear dye, Hoechst 33258 (1μg/ml; Polysciences, Inc.) in the final two washes. Alexa-fluor 568 F-Actin (1:200, Molecular Probes), an actin intermediate filaments dye, was added to washes whenever the antibody combination allowed, avoiding the overlap of the labeled actin (Alexa 568) and the wavelength of the secondary antibody. Ovaries were mounted in 10 µl of 50% glycerol/PBS solution containing sodium azide and Hoechst 33258 (1μg/ml; Polysciences, Inc.), the cover glass was mounted on top of mixture of vasoline: lanolin: paraffin (1:1:1) which was applied at the corners of the coverslip to minimize compression.

All secondary antibodies used were from Molecular probes (Invitrogen, USA; Table I).

Image acquisition and analysis

Whole mount or sectioned ovary preparations were examined with a LSM-5 Pascal confocal microscope (Zeiss, Germany) mounted on a Zeiss Aziovert 200M microscope equipped with Diode laser (405nm), Argon laser (458, 477, 488 and 514nm) and Helium Neon laser (594nm). Single scans or Z series data sets were made using either 20x, 40x, 63x and 100x objectives (Zeiss, NA=1.25) after the necessary adjustments in gain and offset to minimize saturation. Acquired data files were analyzed using LSM Image Examiner software. Bright field images were acquired with a Sony XWaveHAD color camera mounted on a Nikon Eclipse 8i microscope, using objectives 20x and 100x.

Connexin foci and primary cilia counts

Connexin 37 and 43 foci and primary cilia were counted in acquired fluorescence images at 40x(raw), using the LSM Image Examiner software. Counts were carefully done in follicles with the oocyte nucleus in focus, at all follicle stages and mouse ages studied. In connexin 43 foci counting it was also differentiate the foci location within the follicle: at the oocyte surface (Cx43 OO surface), in the first granulosa cell layer (Cx43 1st GC layer), in the second granulosa cell layer (Cx43 2nd GC layer), and from second granulosa cell layer beyond (Cx43 2nd GC layer on). For primary cilia counting, we only counted those associated to a pericentrin foci.

RESULTS

Epithelial and cortical stroma postnatal events

Here we used immunoflourescence analysis of cytoskeleton and ECM components: actin microfilaments, cytokeratins intermediate filaments, heparin sulphate proteoglycans (HSPG) and collagen type IV. Morphological changes undertaken by the ovary during follicle assembly and throughout development are shown in figure 1. It was possible to observe the ovigerous cords continuity with ovarian surface epithelium in fetal (E19.5) and to a lesser extent in neonatal ovaries (P2) (Fig. 1A-B arrow). Fetal and neonatal ovaries had oogonia clusters scattered throughout the ovary, which later began to disassemble giving rise to assembled primordial follicles, some of which could grow into primary follicles (P6) (Fig. 1C arrows). Pre-pubertal (P20) ovaries exhibited a more complex organization, with a clear population of growing follicles in the medulla (Fig. 1D). Cytokeratins were almost exclusively expressed in the ovarian surface (Fig. 1E-H arrows). During cluster fragmentation it could also be detected in the epithelium of the ovigerous cords (Fig. 1E, insert). Note that, at the time of follicle assembly, cytokeratins show a discontinuous pattern (Fig. 1E-F, arrows), contrasting with the continuous tightly delimiting

pattern in ovarian surface epithelium in adult ovaries (Fig. 1H). Regarding ECM markers, we observed HSPG delimited clusters and basally delimited the ovarian surface at E19.5, although the pattern was discontinuous (Fig. 1I, arrowheads).

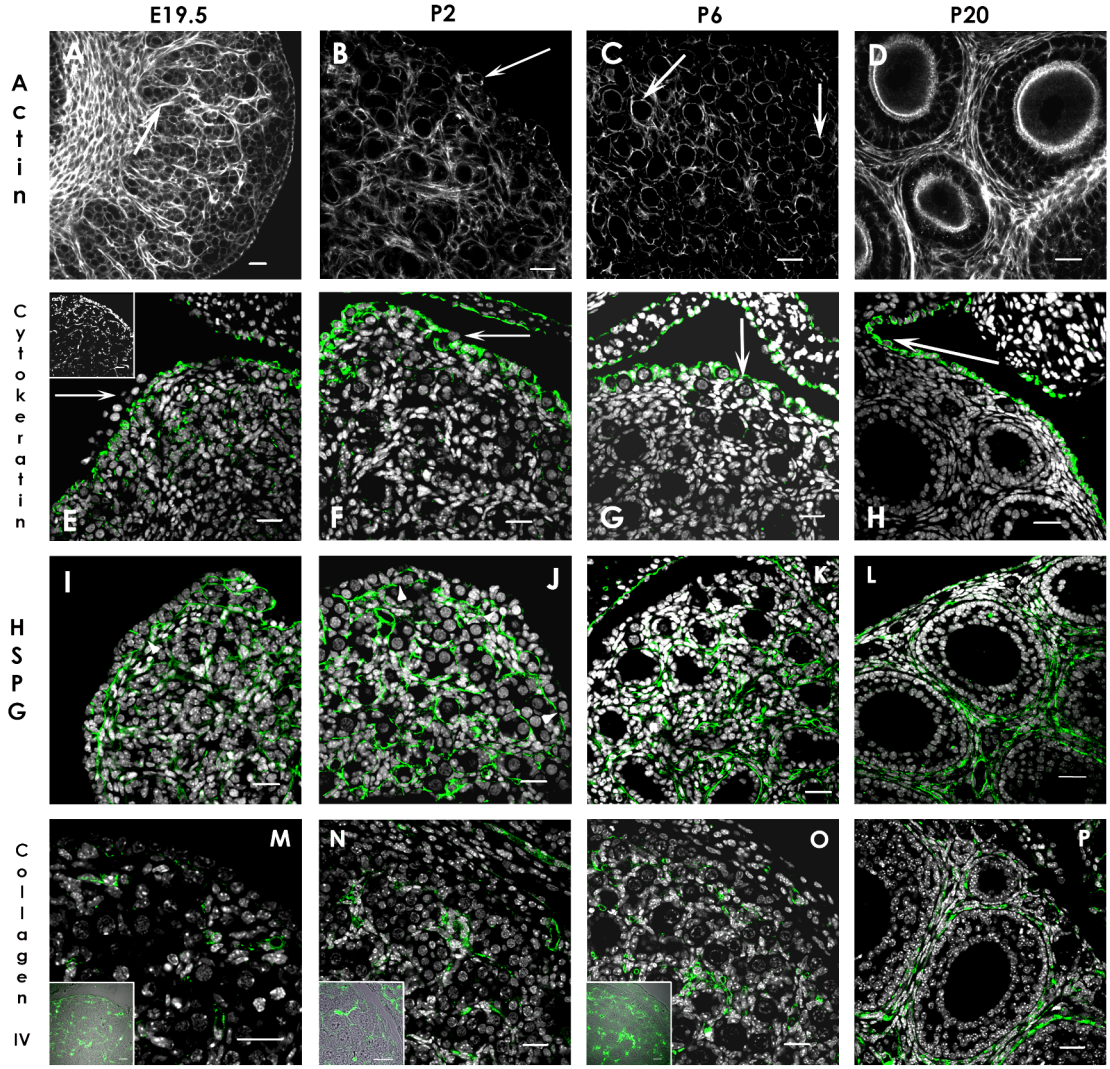

Figure 1 – Overview of ovarian development with cytoskeleton and ECM markers. Actin filaments, cytokeratin, HSPG, collagen type IV, distribution at embryonic day 19.5 (E19.5 – A, E, I and M); postnatal days 2 (P2 – B, F, J and N); postnatal day 6 (P6 – C, G, K and O) and postnatal day 20 (P20 – D, H, L and P). A-D white actin, arrows pointing to ovigerous cords in A, the discontinuity of ovarian surface in B, and primordial follicles in C. E-H green cytokeratin and white nuclei, arrows pointing to the ovarian surface epithelium, E-F note ovarian surface discontinuity; E insert white cytokeratin. I-L HSPG green and white nuclei, I-J arrowheads pointing to ovarian surface gaps. M-P collagen type IV green and white nuclei, M-O inserts green collagen type IV and DIC. Scale bars = 20μm

When clusters initiate fragmentation and start to assemble follicles, HSPG was faintly detected and incompletely delimited the pre-granulosa cells

(Fig. 1I-J, arrowheads). With primordial follicle formation HSPG was basally surrounding the follicles and almost undetected at the ovarian surface (Fig. 1K). In pubertal ovaries HSPG was basally expressed in the ovarian surface epithelium and surrounding theca cells of the growing follicles (Fig. 1L). Type IV collagen was almost undetectable in fetal and neonatal ovaries (Fig. 1M-N, inserts), however it could be seen in growing follicles, at postnatal day 6 (P6), (Fig. 1O, insert). Interestingly, in pubertal ovaries (P20), collagen type IV was delimiting the theca cells (Fig. 1P), but not the ovarian surface epithelium.

The changes observed could also be detected when analyzing the mean number of follicles per ovary plotted in box-whisker (Fig.2).

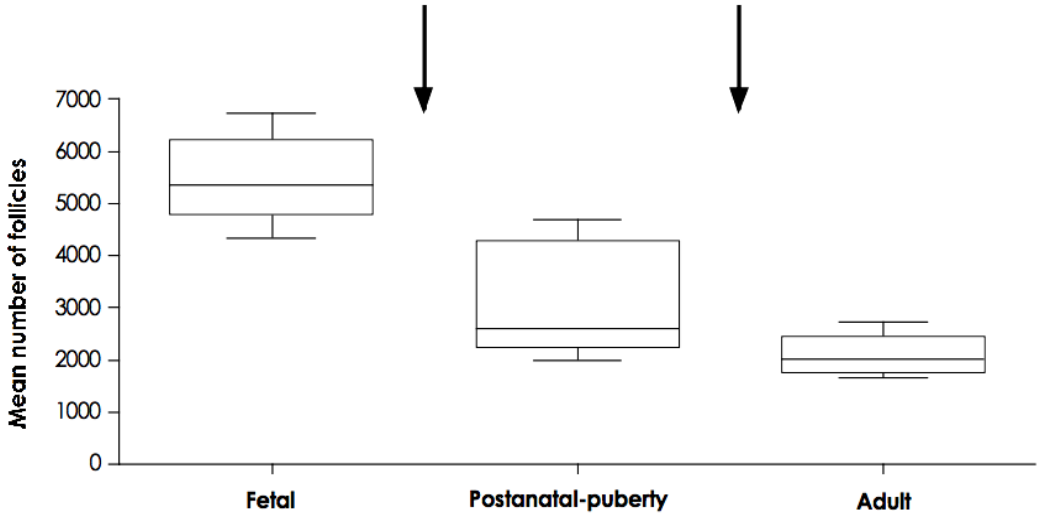

Figure 2 – Mean number of follicles in fetal (E15.5 and E19.5), postnatal to puberty (P2-P20), and adult (P42-P150), note the decrease at birth and when ovulation initiates (arrows). Day of birth: day 20 post coitus.

Emergence of follicular gap-junction parallels follicle assembly

Having evaluated ovarian extracellular matrix changes through ovarian development, we went on to investigate the cell-cell communication, using the markers: β-catenin for cell adhesion and connexins 37 and 43 gap junction expression over the same ovarian developmental stages.

β-Catenin was expressed in the ovary throughout development (Fig. 3A-D). It localized in fetal ovaries, from the oolema to the cell borders of ovarian

epithelium (Fig. 3A). In postnatal ovaries, the oolema of primordial follicles continued to be intensely stained, as well as the granulosa cell membranes of all growing follicles (Fig. 3B-D). Interestingly, the intense staining seen in primordial follicles was also detected in some of the growing follicles (Fig. 3D).

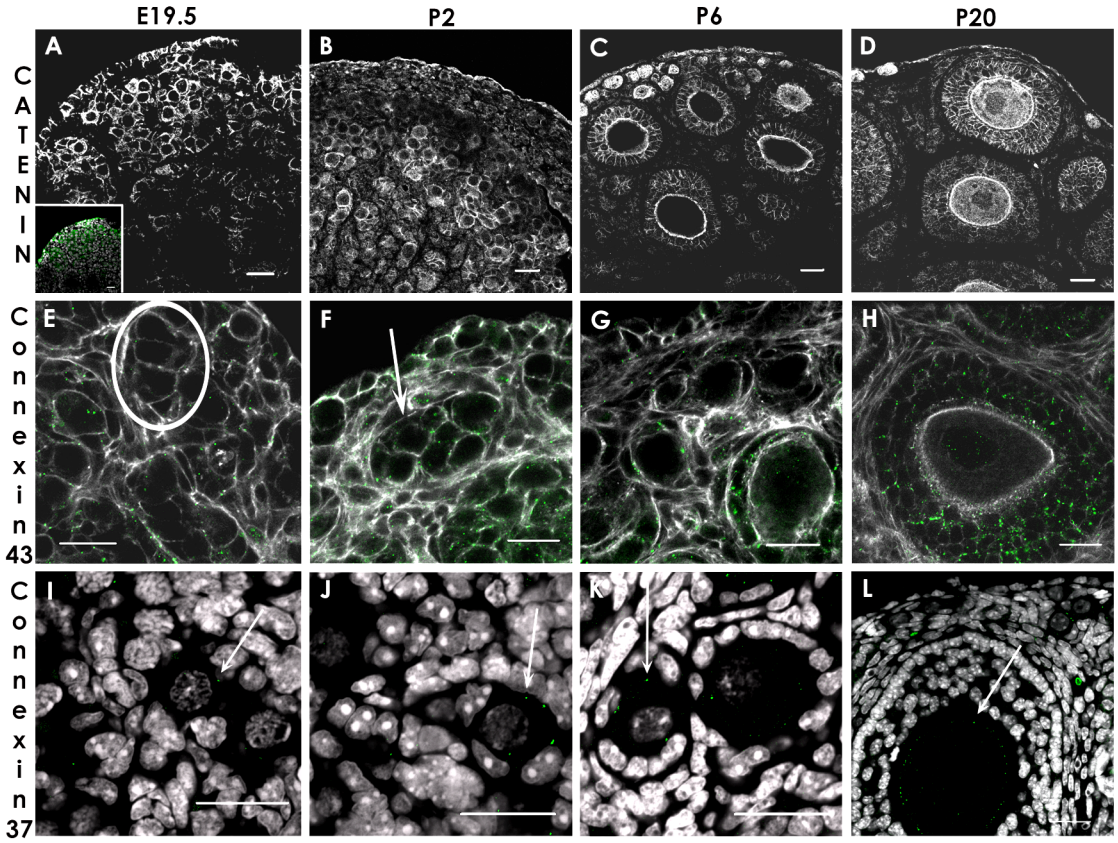

Figure 3 – Adhesion and gap junction development in the mouse ovary. β-Catenin labeling allows to identify the cell contours within the ovary (A-D – white β-catenin, A-insert green β-catenin and white nuclei). β-Catenin is also expressed in the ooplasm of primordial follicles in postnatal ovaries (C-D). Connexin 43, depicting gap junctions, are expressed from embryonic to pubertal ages (E-H – green and white actin; white circle a cluster – E; arrow pointing to a cluster in F). Connexin 43 junctions occur between oocyte and granulosa cells and between granulosa cells, the latest being more abundant. Connexin 37 is restricted to the oolema at all ages and stage follicles (I-L – green and white nuclei). Arrows pointing the connexin foci. Scale bars = 20μm.

Regarding the distribution of connexins, we observed both heterologous (granulosa–oocyte) and homologous (granulosa-granulosa) cell junctions with connexin 43 (Fig. 3E-H). Albeit being seen between oogonia and somatic cells surrounding it at P2 (Fig. 3F arrow), cx43 was also seen between the somatic cells part of the ovigerous cord forming the clusters

(Fig. 3E circle-F arrow). Homologous cx43 expression continues to increase in granulosa cells of secondary follicles (Fig. 3G-H and 4 (from 2nd GC layer and on)). In general, cx43 expression increased with age/follicle growth, and was higher in the ovary medulla. It was detected in the ovarian surface epithelium only at P20.

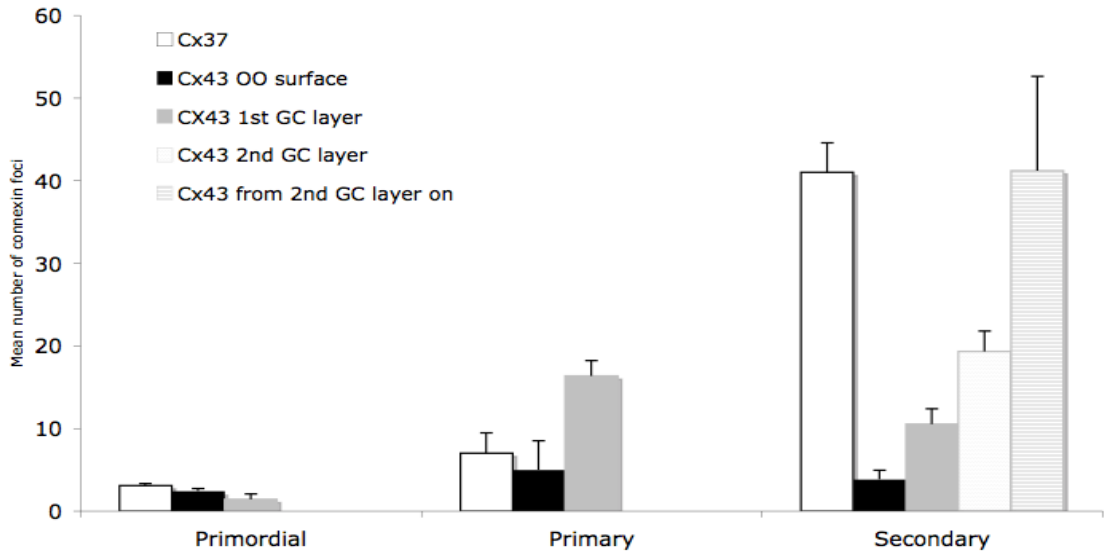

Figure 4 – Mean number of cx37 and cx43 foci distribution through follicle stage- (primordial, primary and secondary follicles). Connexin 43 foci were counted according four different locations: oocyte surface (Cx43 OO surface); first granulosa cells layer (Cx43 1st GC layer); second granulosa cells layer (Cx43 2nd GC layer); and from second granulosa cells layer until theca (Cx43 from 2nd GC layer on). (n=4-10 images per age; error bars=SEM)

Connexin 37 expression also increased with follicle growth, but its expression was restricted between oogonia and their surrounding somatic cells and between the oocytes and neighboring granulosa cells (Fig. 3I-L). In clusters at E19.5, it was possible to observe at least one cx37 foci per oogonia (Fig. 3I), as opposed to the higher number observed at P20 (Fig. 3L and 4). Interestingly cx37 (a germ cell specific connexin) seems to occur independently of age.

The expression of both connexins is an indication of early bi-directional communication between oocyte and granulosa cells. To better evaluate connexin expression we counted the number of cx37 and cx43 foci within primordial, primary and secondary follicles. Interestingly, the number of

double labeled foci increased with follicle growth (Fig. 4), while cx43 foci within the ooplasm (cx43 OO surface) were relatively constant despite oocyte and follicle growth (Fig. 4).

These results demonstrate the early establishment of somatic-germ cell direct interactions and its maintenance seems to occur independent of where or how primordial follicles are formed.

True ovarian surface epithelium and follicle assembly: are primary cilia and Notch involved?

Since Notch has been implicated in germ-somatic cell communication (Zhang *et al*., 2011), we compared actin and tubulin (cytoskeleton markers) with Notch2 distribution at the specific time of follicle assembly.

We observed tubulin going from lace-like to a cortical band separating the true epithelium from the underlining germ and somatic cells (Fig. 5D-I). This is accompanied by Notch2 distribution in granulosa cells near the oolema and in the ooplasm at all stages of follicle growth (Fig. 5A-F). As expected from a transmembrane receptor protein, Notch2 showed dotted/foci-like distribution. Its intensity seemed to increase with follicle/oocyte size, especially in the ooplasm (Fig. 5A-F). Generally Notch2 expression was higher in the ovary medulla when compared with cortical area, similar to the pattern of connexin43 (Fig. 3E-H). Since our anti-Tubulin antibody was specific to acetylated-tubulin, it was also possible to identify a very stable structure: primary cilia. To ensure that what we were seeing was in fact primary cilia, we also labelled ovarian sections for pericentrin, a centrosome marker (Fig. 5G-I). Primary cilia can be seen among the lace-like microtubules as short, thick very oriented structure in green enucleating from a small red dot – the centrosome (pericentrin) (Fig. 5G-I arrows). Primary cilia were often found on somatic cells, often next to germ cells, and occasionally very close to the oolema, lying parallel to the oocyte (Fig. 5G-H arrows). Interestingly, some of these primary cilia had a Notch2

foci associated, especially during embryonic ages (Fig. 5D arrowheads), but even at birth (P1) (data not shown).

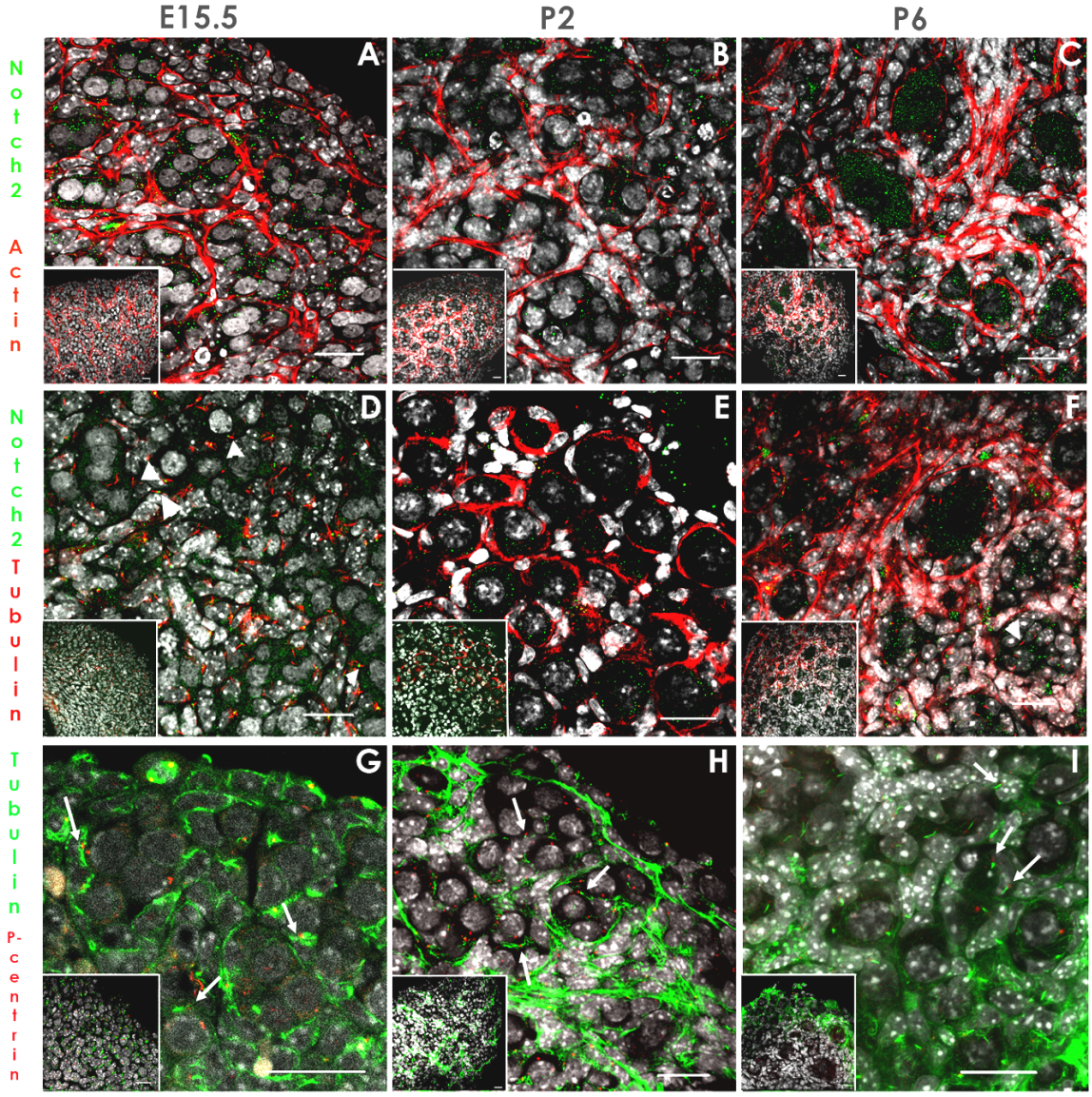

Figure 5 – Notch distribution and primary cilia occurrence during follicle assembly. Notch foci expression in embryonic and neonatal ovaries (A-C – green Notch2, red actin and white nuclei; D-F – green Notch2, red tubulin and white nuclei). Acetylated-tubulin is expressed in embryonic and primordial ovaries in a lace-like pattern and in a thicker structural-like pattern in growing follicles (D-I; G-I green tubulin, red pericentrin and white nuclei). At all ages and follicle stages is possible to observe a small thick tubulin structure preceded by a red centrosome (pericentrin; G-I arrows). Occasionally these are preceded by a Notch2 dot in embryonic ovaries (D arrowheads). Scale bars = 20μm.

The mean number of primary cilia increases with follicle stage, consistent with its distribution mostly in granulosa cells (Fig. 6). These results may

suggest a sensory role of primary cilia in the germ-somatic cells interface associated to the Notch pathway.

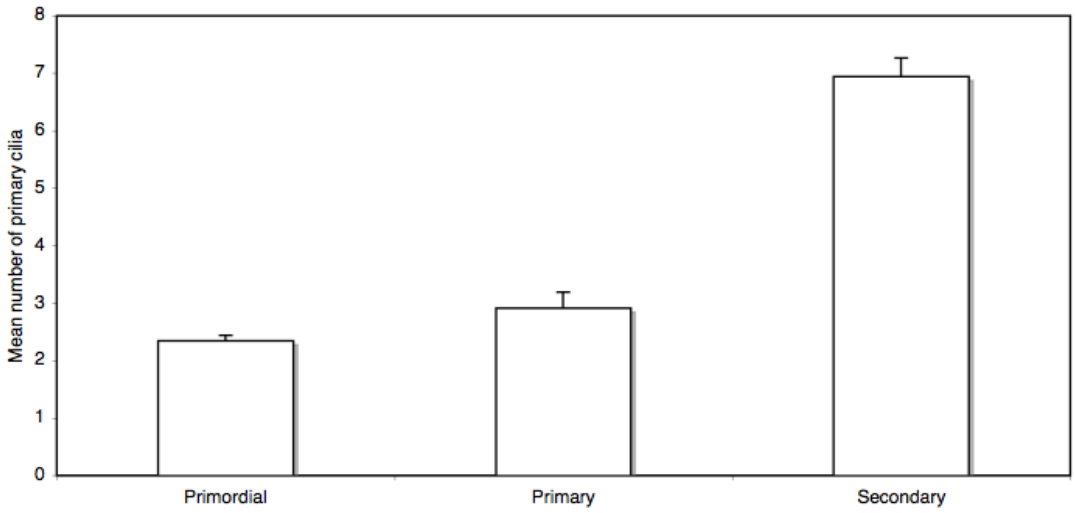

Figure 6 – Mean number of primary cilia per follicle stage. (n=6-16 images per age; error bars=SEM)

DISCUSSION

The present study shows that ovarian follicle morphogenesis is the result of coordinated germ-somatic-matrix cell interplay, which results in the formation of the follicular reserve shortly after birth in the mouse.

The adult ovary is a complex organ with the stroma divided into the cortex and medulla, surrounded by an epithelium, and regulated by gonadotropins and steroid homones (McGee and Hsueh, 2000; Sathananthan *et al.*, 2006). The functional unit of the mammalian ovary is the follicle comprised of theca and granulosa cells, and oocytes. During ovarian histogenesis the germ cells are grouped within epithelial ovigerous cords, which are fundamental for follicle formation, since no follicle formation occurs in the absence ovigerous cords (Byskov *et al.*, 1977). However, the opposite is not true, without oogonia the ovigerous cords still form (Byskov *et al.*, 1977; Mazaud *et al.*, 2005). In the mouse, follicle formation occurs shortly after birth, with the fragmentation of ovigerous cords and the enclosing of oocytes by pre-granulosa cells, somatic cells from the ovigerous cords, resulting in the formation of the primordial follicles. Cluster fragmentation is a result of basal membrane remodeling,

which involves cytoskeleton and cellular adhesion alterations (Mazaud *et al*., 2005). Interestingly, a recent report unveiled more complexity of granulosa cell specification in the mouse (Mork *et al*., 2012). These authors showed that pre-granulosa cells enclosing the medullary follicles those primordial that activate growth immediately after birth are derive from an apparently different cell lineage that the granulosa cells that enclose the cortical primordial follicles which remain arrested until they are activate later in the adult ovary. Interestingly, another recent report showed that granulosa and ovarian surface epithelial cells have a common ancestor, the precursor cells from the mesonephros (primitive kidney) (Hummitzsch *et al*., 2013). Both reports are in accord with the hypothesis, first proposed by Hirshfield (1992) of two different types of primordial follicles. This hypothesis was recently proven by two independent studies. Zheng and Zhang and co-workers (Zheng *et al*., 2014), using tomoxifen-inducible reporter mouse model, *Foxl2CreER^{T2};mT/mG*, traced granulosa cells from two different precursor cells (Zheng *et al*., 2014). In the same line of evidence, Liu and colleagues (Liu *et al*., 2015) demonstrate that theca cells have two distinct progenitor cells. These cells derived from fetal ovary and mesenchyme acquiring theca cell lineage by expressing Gli1, in response to paracrine signal of oocyte-granulosa cells communication (Liu *et al*., 2015).

Here we found that the interplay of germ-somatic cell is a key regulator of follicular assembly and architectural changes favoring ovarian reserve establishment. Possibly, this interplay is selecting germ cells, those that would become part of the early-activated primordial follicles and those that will become part of the dynamic follicular reserve at puberty.

Oogonia cluster breakdown

We observed ovarian morphological changes throughout development; these were accompanied by the two major periods of germ cell loss – the onset of follicle assembly and of the approach to ovulation. Neonatal germ cell loss was previously studied by us (Rodrigues *et al*., 2009) and here

we described how cytoskeleton, extracellular matrix and the epithelium coordinate in order favor follicle assembly.

Actin, a cytoskeleton filament, demonstrated the morphological changes from germ cell clusters (E19.5) to follicle assembly, with oogonia clusters giving rise to primordial follicles (P2 and P6), which then grew into primary follicles and later (P20) a population of growing follicles in the medulla could be observed. This observation is consistent with "simultaneous primordial follicle activation" (Zheng *et al*., 2014; Liu *et al*., 2015). We've also investigated cytokeratin distribution, an intermediate filament characteristic of epithelial cells (Duprey *et al*., 1985; McLean and Lane, 1995; Plancha, 1996). Here a broad-range antibody was used, which immunolocalized to the outer limits of the oogonial clusters at E19.5, showing that ovigerous cords presented an epithelium. Cytokeratin labelling became more restricted to the ovarian surface epithelium by postnatal day 6 when the ovigerous cords/clusters had broken-down to assemble primordial follicles. This could be evidence for the need of a true epithelium in order to restrict the shedding of germ cells, thus allowing the establishment of the follicular reserve. Fridmacher and colleagues (Fridmacher *et al*., 1992) also described this expression pattern in the rat, using a broad-range cytokeratin antibody and specific antibodies to cytokeratins 8, 18, and 19. The same specific cytokeratins were found in the ovigerous cords of rat, pig (Czernobilsky *et al*., 1985) and human fetal ovaries (Czernobilsky *et al*., 1985; Benjamin *et al*., 1987). Our embryonic staining also suggested the continuity of the open end of the ovigerous cords with the ovarian surface epithelium (Fig. 1 A and B)(Byskov and Lintern-Moore, 1973; Sawyer *et al*., 2002; van den Hurk and Zhao, 2005), which may again contribute to germ cell shedding while follicle assemble, and ultimately to early activated primordial follicle reserve establishment (Monniaux *et al*., 2014).

Coincident with the cytoskeleton rearrangements of the ovary was the distribution of the ECM component, HSPG, a proteoglycan that adjusts its

basal localization from delimiting clusters to theca cells. The discontinuous distribution of HSPG at the ovarian surface epithelium prior and during follicle assembly (ovigerous cords fragmentation), as implicated by cytokeratin, suggests that the ovigerous cords were still open at the end apposed to the ovarian epithelium. This also occurs in the embryonic ovaries of rat (Rajah *et al*., 1992) and sheep (Sawyer *et al*., 2002). The interaction of proteoglycans like HSPG with cytoskeleton elements, through membrane molecules, facilitates ECM adhesion (rat embryo fibroblast: (Woods *et al*., 1985); ECM and rat granulosa cells: (Ben-Ze'ev and Amsterdam, 1986; review: Bishop *et al*., 2007). Moreover, HSPG also seem to interact with other ECM components, as type IV collagen, as our results indicate. Type IV collagen is the network-forming collagen, which assemble sheet-like fibers (basal membranes) within the extracellular matrix (Irving-Rodgers *et al*., 2006). We observed type IV collagen primarily within the stromal cells surrounding the oogonial clusters in embryonic and neonatal ovaries, consistent with facilitating germ cell loss during follicle formation and establishing follicular reserve. Our results are in agreement with those from Berkholtz and colleagues (Berkholtz *et al*., 2006), which encounter type IV collagen only faintly in primary follicles. In rat, it has also been reported the expression of type IV collagen bordering ovigerous cords in ovaries at 18.5 days post *coitus*, a day before our most early age (E19.5) (Mazaud *et al*., 2005; and on day 1 Rajah *et al*., 1992 (using aniline blue); Rajah and Sundaram, 1994). However, even though using the same antibody, Pan and Auersperg (1998), reported different results with rat ovaries, like cytokeratins, type IV collagen diminish with follicular maturation. This difference may be due to the fixation method (acetone in frozen sections and here Bouin's fixative in paraffin sections), or the animal model (strain of rat).

Recently, it was shown that the presence not only of collagen type IV but also type XVIII, perclan and nidogen 1 and 2 in the basal lamina between stroma and ovigerous cords (Hummitzsch *et al*., 2013). The authors

suggested that the penetrating stroma helps in compartmentalization and formation of follicles and ovarian surface epithelium (Hummitzsch *et al*., 2013). This is in agreement with our observations of ECM markers and cytoskeleton changes throughout development.

We have also investigated adhesion and gap junction cell interactions, using: β-catenin and connexins 37 and 43. β-Catenin was expressed uniformly throughout development, from oolema to ovary surface cell borders, in fetal ovaries and postnatal, and was in all ovarian compartments. Gap junctions are intracellular membrane channels that allow direct communication and exchange of cytoplasmic molecules, such as second messengers and small metabolites between neighboring cells (Bruzzone, 2001). They are fundamental for correct development, as suggested by the absence of germ cells and neonatal death of mice lacking connexin 43 due to heart malformations (Juneja *et al*., 1999). Here we have studied the distribution of connexins 37 and 43. Connexin 37 was expressed in junctions between the granulosa cells and the oocyte, consistent with previous reports (Simon *et al*., 1997; Wright *et al*., 2001; Veitch *et al*., 2004; Teilmann, 2005; Simon *et al*., 2006). Here we have also observed heterologous gap junctions between germ cells within clusters and the somatic cells that constitute the wall of the ovigerous cords. This reinforces the proposal that some somatic cells of the ovigerous cord are pre-granulosa cells. Mitchell and Burghardt (Mitchell and Burghardt, 1986) first describe gap junctions in ultra-structural studies with embryonic mouse ovaries starting at 13.5 days post coitum. Here we show for the first time that connexin 37 is part of these gap junctions from at least E19.5. Like connexin 37, connexin 43 was detected from embryonic to neonatal between granulosa cells of all stage follicles present, again in accordance with other reports (Juneja *et al*., 1999; Ackert *et al*., 2001; Wright *et al*., 2001; Gittens *et al*., 2003; Teilmann, 2005). Connexin 43 was observed in gap junctions within the embryonic ovary, confirming the Mitchell and Burghardt (Mitchell and Burghardt, 1986) observations, but contrasting with

those of Mayerhofer and Garfield (Mayerhofer and Garfield, 1995). Nevertheless our results are in agreement with those of Pérez-Armendariz (2003), who studied connexin 43 expression in the fetal mouse ovary. The authors also observed heterologous connexin 43 gap junctions between the germ cells and the somatic cells of the ovigerous cords. Again these observations reinforce the importance of gap junction in follicle formation and how the somatic cells that exhibit these junctions are most likely precursor of pregranulosa cells. Moreover, the highest incidence in the ovary medulla may be an evidence of Mork and colleagues (Mork *et al*., 2012) findings. These authors reported that cells from the bipotential gonad are precursors of granulosa cells of the primordial follicles in the medulla of the ovary, which initiate growth immediately after birth (Mork *et al*., 2012). Also in agreement with Zheng colleagues (Zheng *et al*., 2014) finding of two primordial follicle classes. One, are the medullary follicles that are activated immediately after birth, which contribute to the onset of puberty (Zheng *et al*., 2014). And the other class of primordial follicles, are those formed after birth, located in the ovarian cortex, which become the second primordial follicle reserve from 3 month of age and later (Zheng *et al*., 2014; reviewed Monniaux *et al*., 2014).

Hence the increased medullary connexin 43 expression before birth may be the associated with early selection of those pre-granulosa cells.

In proliferating cells gap junctions have also been proposed as survival and death signals (Krysko *et al*., 2004; Krysko *et al*., 2005). Connexin 43 has been specifically implicated in granulosa cells survival (Krysko *et al*., 2004). This is in accordance with follicle formation during cluster breakdown, when some somatic cells surrounding the germ cells become the pre-granulosa cells of the newly formed primordial follicles (Pepling and Spradling, 1998). Perhaps those somatic cells that have more connexin 43 foci will be selected as pre-granulosa cells.

In sum, our observations point to matrix-somatic cell interactions in

conjunction with cytoskeleton, adhesion and gap junction molecules, as major players in follicle assembly, simultaneously facilitating early germ cell escape through the ovarian surface due to a discontinuous epithelium, thus contributing to establishing the reserve of the firstly activated class of primordial follicles.

Architectural changes and follicular reserve establishment

Until now we have pinpointed and discussed cellular arrangements first during cluster breakdown and second during follicle formation and growth, which together contribute to follicular reserve establishment. With the aim to contribute to a detailed description of ovarian morphogenesis, we also examined how the cytoskeleton and Notch signaling influence these arrangements and the ovarian architecture.

The cytoskeleton is responsible for cell morphology, shape and movement, (Ben-Ze'ev and Amsterdam, 1986); (Usui, 2007). Microtubules and actin filaments are two of the main protein components of cytoskeleton; they are assembled by polymerization and depolymerization, making these proteins very dynamic (Usui, 2007).

The distribution of tubulin was lace-like within the clusters (at E19.5) and remained throughout development into adulthood in primordial follicles, changing to a thicker pattern with follicle growth. Recently, Mora and colleagues (Mora *et al*., 2012) reported a lack of α-tubulin organization in primordial follicles, but it was abundant and running parallel along the cell axis in granulosa cells, very similar to what we saw with acetylated α-tubulin.

The Notch signaling pathway is highly conserved throughout vertebrates, and involved in cell proliferation, differentiation and apoptosis (Artavanis-Tsakonas *et al*., 1999). Recently, Zhang and co-workers (Zhang *et al*., 2011) added Notch signaling inhibitors to a primary follicle culture and observed developmental arrest and granulosa cell detachment. Trombly and colleagues (Trombly *et al*., 2009), showed the presence Notch2 in germ

cells prior to follicle assembly. We found a similar pattern, which is consistent with the adult distribution as reported by Johnson and co-workers (Johnson *et al*., 2001). We found that Notch2 expression increased with follicle growth, and was also higher expressed within the ovarian medulla. Most interesting was the observation of Notch2 associated with primary cilia in perinatal ovaries. These observations strongly suggest an involvement of cytoskeleton and cell communication in ovarian development. Primary cilia are involved in sensing of mechanical and chemical cues during development, including Notch signaling (reviewed (Seeger-Nukpezah and Golemis, 2012). Therefore, the primary cilia may be an early precursor of the tranzonal processes (TZP's), which connect granulosa cells to the oolema of growing follicles, thus reinforcing the importance of granulosa-germ cell communication. It is also possible that the primary cilia, together with gap junctions (connexin 43 results) and Notch signaling may be involved in the early selection of those germ-somatic cells that will become part of the first follicle population to be activated soon after birth. As it as been shown the granulosa cells have different precursor cells (Hirshfield, 1992; Mork *et al*., 2012; Hummitzsch *et al*., 2013), those that will enclose the first follicles activated are located in the medulla, which is coincident with the observed increased expression of connexin 43 and Notch. Interestingly, anti-mullerian hormone (AMH) seems to regulate the express of notch receptor, Jagged by mediating stromal-epithelial interaction (Nilsson *et al*., 2011). Our results show well this stromal-epithelial-germ cell interactions.

Taken together these results show how germ-somatic-matrix communication coordinates cytoskeleton and ECM via adhesion/gap junctions and Notch signaling inducing ovarian ontogeny and follicular reserve.

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Table I – Primary and secondary antibodies combinations in paraffin section and whole ovary immunofluorescence.

Primary Antibody	Secondary Antibody	Dye	Observations
Acetylated-Tubulin (1:100, Sigma)	Alexa-fluor 488 (1:800, Molecular Probes) Alexa-fluor 568 (1:800, Molecular Probes)	Hoechst (1 µg/ml; Polysciences, Inc.)	Microtubule marker used in paraffin sections and whole ovaries. Paraffin sections fixed in PFA and antigen retrieved; whole mounts fixed in MTSB-XF.
Heparin Sulphate ProteoGlycan – HSPG (1:100)	Alexa-fluor 488 (1:800, Molecular Probes)	Eth-D2 (1:100; Molecular Probes)	Extra cellular matrix marker done in paraffin sections fixed in PFA, antigen retrieved.
Collagen type IV (1:100, Southern Biotech)	Alexa-fluor 488 (1:800, Molecular Probes)	Eth-D2 (1:100; Molecular Probes)	Extracellular matrix, basal lamina marker done in paraffin section fixed in Bouin's, antigen retrieved.
Pan-Cytokeratin (1:100, Sigma)	Alexa-fluor 488 (1:800, Molecular Probes)	Eth-D2 (1:100; Molecular Probes)	Intermediate filament, basal membrane marker done in paraffin section fixed in PFA, antigen retrieved. Broad-range antibody recognizing cytokeratins 1, 4, 5, 6, 8, 10, 13, 18 and 19.
ß-catenin (1:100, Transduction)	Alexa-fluor 488 (1:800, Molecular Probes)	Eth-D2 (1:100; Molecular Probes)	Junctional marker used in paraffin sections fixed in PFA, antigen retrieved.
E-Cadherin (1:100, Sigma)	Alexa-fluor 488 (1:800, Molecular Probes)	Eth-D2 (1:100; Molecular Probes)	Epithelial marker used in paraffin sections.
Connexin 37 (1:100; Alpha Diagnostics)	Alexa-fluor 488 (1:800, Molecular Probes)	Eth-D2 (1:100; Molecular Probes)	Gap-junctional oocyte specific marker; paraffin sections fixed in PFA.
Connexin 43 (1:100; Santa Cruz)	Alexa-fluor 488 (1:800, Molecular Probes)	Eth-D2 (1:100; Molecular Probes)	Gap-junctional marker; paraffin sections fixed in PFA.
Notch 2 (1:100)	Alexa-fluor 488 (1:800, Molecular Probes)	Eth-D2 (1:100; Molecular Probes)	Transmembrane receptor, part of a signaling pathway involved in cell proliferation, differentiation and apoptosis. This antibody in particular recognizes the external part of the receptor.

Alexa-fluor 568 F-Actin (1:200, Molecular Probes), dye used is whole mounts for staining actin intermediate filaments.

CHAPTER # 4

Oocyte and hormonal regulation of the ovarian follicle reserve in the mouse

(Patricia Rodrigues, Darlene Limback, Lynda McGinnis, T Rajendra Kumar, Martin Matzuk, David F. Albertini1and Carlos E. Plancha – manuscript in preparation for submission)

ABSTRACT

Purpose: We have previously shown that perinatal ovarian remodeling determines ovarian follicle reserve establishment. In this study we wanted to discriminate between germ cell and hormonal factors involved in this process, using specific gene deficient mouse models.

Methods: GDF9 (growth differentiation factor 9), Nobox (newborn ovary homeobox gene), Sohlh1 (spermatogenesis and oogenesis basic helix-loop-helix 1), and FSHβ (follicle stimulating hormone) deficient mouse models were used in this study. Immunofluorescence microscopy was used to monitor cytoskeletal, extracellular matrix (ECM), and cell junction phenotypes using whole mounts and paraffin sections of newborn or adult ovaries. In addition, GDF9 mRNA and protein in 21 days old females was evaluated by *in situ* hybridyzation (mRNA) and fluorescence labeling (protein) in paraffin sections. High-resolution confocal microscopy was used to monitor cytoplasmic and organellar acidification with Lysotracker Red (LTR) in Nobox and Sohlh1 newborn ovaries to ascertain various forms of programmed cell death.

Results: Deficiencies in germ and somatic cell interactions were observed in both FSHβ and GDF9 knockout models that coincided spatially and temporally with alterations in the cytoskeleton, ECM and adherent junction. All knockout models exhibited disorganized stromal and epithelial

properties when compared with age matched control ovaries from C57/Bl6 or CD-1 wild type mouse strains. With respect to cell death pathways, Nobox and Sohlh1 ovaries, exhibited prominent signs of both apoptosis and autophagy in germ cell lineages but evidence of somatic cell loss was most apparent in the Sohlh1 knockout model, indicating that this gene, may be preferential impacting somatic cells.
Conclusions: Germ-somatic cell interactions are modulated by both oocyte-intrinsic (GDF9, Nobox) and ovary extrinsic (FSHβ) factors that appear to influence patterns of stromal and epithelial remodeling associated with ovarian development.

INTRODUCTION

Despite its heterogeneous tissue composition, the ovary is an extremely well organized organ. The interactions between germ cells (oogonia and oocytes), and somatic cells (granulosa, theca and stromal cells), vasculature, and extracelular matrix (ECM) are essential in some capacity to achieve follicle formation, growth, ovulation and luteinization. These interactions are regulated by a number of factors (Richards, 2001), both locally produced and from distant origins, such as the hypothalamic-pituitary-ovary axis (Gougeon, 1996; McGee and Hsueh, 2000). Well known adult ovary events, such as antral follicle growth, maturation, ovulation and lutenization are controlled by two hormones: Follicle Stimulating Hormone (FSH) and Luteinizing Hormone (LH), secreted by the pituitary gland, which in turn is controlled by Gonadotropin-Releasing Hormone (GnRH), produced in the hypothalamus (Gougeon, 1996; McGee and Hsueh, 2000). FSH receptors (FSHr) are localized in granulosa cells in the ovary (Kumar *et al*., 1997; Kumar *et al*., 1999; Burns *et al*., 2001), belonging to a superfamily of transmembrane receptors coupled to G-proteins (Oktay *et al*., 1997). Primordial follicle recruitment and the first phases of follicle development are independent of FSH, as evidenced by the presence of preantral follicles in α-GSU deficient female mice (the common glycoprotein α-

subunit to the pituitary and placental glycoprotein hormone family), as well as in FSHβ-deficient female mice (Kumar, 2005). Nevertheless, recently it has been suggested that FSH promotes primordial follicle formation in mice through stimulation of activin (Lei *et al*., 2010).

Growth differentiation factor 9 (GDF9) a member of the transforming growth factor β (TGF-β) super family that during development mediate several cell behaviors, including proliferation, differentiation, extracellular matrix production, and cell death (Pangas and Matzuk, 2004), has also been implicated in follicle growth. GDF9 is a germ cell specific factor was previously found expressed in growing follicles but not in the quiescent primordial/primary follicles in the mouse (McGrath *et al*., 1995; Elvin *et al*., 1999a). However, Wang and Roy (2006) reported the importance of endogenous GDF9 for follicle formation, in hamster. Moreover, the authors show how FSH regulates GDF9, implying oocyte regulation by FSH in primordial follicle formation. The importance of this growth factor during early folliculogenesis was first demonstrated when Dong and co-workers inactivated the mouse GDF9 gene (Dong *et al*., 1996). Female mice lacking a functional GDF9 gene are infertile due to folliculogenesis arrest at the primary follicle stage (Dong *et al*., 1996; Elvin *et al*., 1999a; Elvin *et al*., 1999b). A more detailed analysis of oocyte and follicle development in these GDF9 deficient females, revealed abnormalities in oocyte-granulosa cell interaction, where an invasion of the periviteline space by the transzonal processes (TZPs) lead to oocyte death (Carabatsos *et al*., 1998). Using RNAi technology it has been suggested that GDF9 is also involved in cumulus cell expansion, (Gui and Joyce, 2005), and is required for Desert hedgehog (*Dhh*) and Indian hedgehog (*Ihh*) production in granulosa cells which are important signals for theca cell differentiation (Liu *et al*., 2015).

Nobox (Newborn Ovary Homeobox) is an oocyte-specific homeobox gene expressed in germ cell clusters, and in primordial and growing follicles (Suzumori *et al*., 2002; Rajkovic *et al*., 2004). Nobox was initially considered a good candidate for oocyte specific factor controlling follicular reserve,

since in the absence of *Nobox*, there is an accelerated postnatal oocyte loss (Suzumori *et al*., 2002). Mutations in the Nobox gene are associated with premature ovarian failure, and also with a delay in germ cell cluster breakdown (Rajkovic *et al*., 2004; Qin *et al*., 2007; Lei *et al*., 2010b).

Nobox is up-regulated by another germ-cell specific gene, Sohlh1, a basic helix-loop-helix transcription factor, critical for early oogenesis (Pangas *et al*., 2006). Sohlh1 disruption causes down-regulation of Nobox and Figla (Factor in the germ-line alpha), both critical in retaining fertility (Pangas *et al*., 2006; Pangas and Rajkovic, 2006).

In the present study we took advantage of mutant mouse lines as a experimental tool to allow better understanding the function of differentially silenced genes (Roy and Matzuk, 2006). Specifically, we used mice with targeted disruption on *FSHβ, GDF9*, *Nobox*, and *Sohlh1*. We demonstrate the importance of oocyte-somatic cells interaction for ovarian histogenesis, and propose a novel mechanism for follicle assembly.

MATERIALS AND METHODS

Animals

Inbred C57Bl/6 and CD1 mice were purchased to Charles River Laboratories, O'Fallon, MO, or Taconic Farms Inc., Germantown, NY. For FSHβ-deficient females we used the progeny of 2 founder pairs heterozygous for FSHβ gene deletion (FSH KO), which were a generous gift of Dr. T. Rajendra Kumar from University of Kansas Medical Center (KUMC). The GDF9 deficient mice (GDF9 KO) were also from our own colony, started with 4 founder pairs that were a generous gift of Dr. M. Matzuk from Baylor College of Medicine, Houston Texas. Dr. M. Matzuk, also generously, shipped us four pairs of newborn (P0) ovaries of each deficient mouse models: Nobox (Nobox KO) and Solhl1 (Solhl1 KO).

All animals were housed in a 14h light: 10h dark environment at constant temperature. Food and water was provided *ad libitum*. Mice were

maintained and used in accordance with the policies of the University of Kansas Institutional Animal Care and Use Committee (IACUC).

Ovary collection and tissue preparation

Females were euthanized by cervical dislocation. After euthanasia ovaries were dissected from female fetuses at fetal 15.5. Some pregnant females were allowed to deliver, which occurred on the early morning of day 20-post coitum. Females were euthanized at days 2, 6, 10, 16 and 42. At least three ovaries per age were collected; the fetal ovaries were collected from at least 2 different pregnant females and the post-birth females from at least 2 different litters. Some females were "primed" at 21 days by injecting them intraperitonially with 5IU equine chorionic gonadotropin (eCG) to recruit or maintain growing antral follicles. Primed females were euthanized at 48 hours post-eCG. Right and left ovaries from each animal were either fixed in Bouin's fluid (Sigma, St. Louis, USA) or 2% paraformaldehyde (PFA, Sigma), for 4-6 hours at room temperature and overnight at 4ºC, respectively. Following fixation, ovaries were transferred to 70% ethanol and processed for paraffin embedding (processor Shandon, GMI, St Paul, USA).

Cell acidification detection

LysoTracker Red (LTR) is an aldehyde-fixable dye that concentrates in acidic membrane-bound intracellular compartments of living tissue, which has been adopted from Zucker and colleagues (Zucker *et al*., 1999) as an assay for programmed cell death in rodent tissues. Here we used a previously described (Rodrigues *et al*., 2009) modified protocol. Briefly, intact ovaries were incubated in 5µM LTR (Molecular Probes-Invitrogen) in MEM (Gibco-Invitrogen) for 1h to 2h at 37ºC and 5% CO_2. Washed twice in PBS and fixed in 4% PFA + 1% glutaraldehyde (Sigma) in PBS (2h; 37ºC with shaking, followed by 4º C, overnight), rinsed twice in PBS and dehydrated in a series of methanol/PBS solutions (50%, 70%, 95% and 100%- 2X; 15

minutes each). Afterwards, tissues were cleared in a 1:2 mixture of Benzyl Alcohol (Sigma) to Benzyl Benzoate (Sigma - BABB). From absolute methanol, tissues were exchanged through 50% and 70% BABB-methanol and finally pure BABB solution (2h each; at room temperature with constant agitation). For confocal microscopy, a single ovary was centrally positioned in a metal washer sealed by Permount (Fisher) to the center of a glass bottomed tissue culture dish (Delta T, Fisher). The depression was filled with BABB and a coverslip applied with Permount (Fisher).

Immunocytochemistry

For follicle counts slides with 5µm sections were dewaxed and re-hydrated using conventional methods. Endogenous peroxidases were quenched in 0,3% hydrogen peroxide in methanol (5 minutes, room temperature). After three washes in Automation Buffer (BiØmeda-Fisher), slides were blocked for 30 minutes at room temperature in 9% goat serum (Zymed) containing 3% Bovine Serum Albumin (BSA, Sigma). Sections were labeled (overnight, 4°C) with polyclonal rabbit antibody against mouse vasa homolog (MVH, gift from Dr. Noce) diluted 1:1200 in 1% BSA. Washed sections were then incubated with biotinylated goat anti-rabbit serum (Zymed; 1:200 in 1% BSA) for 30 minutes at room temperature, rinsed and incubated with Horseradish Peroxidase Avidin D (HRP; Vector; 1:500) for 10 minutes. Diaminobenzidine was used as a substrate for HRP. Sections were counterstained with periodic acid-Schiff's reagent and Harris hematoxylin (Protocol) containing 4% acetic acid. Tissues were dehydrated, cleared and coverslipped using Permount (Fisher). Control slides were processed identically but primary antibody was omitted.

Immunofluorescence

Paraffin sections

Slides were dewaxed and re-hydrated using conventional methods. Antigen retrieval was preformed, to those antibodies that needed it (see

Table I), in a microwave for 15 minutes in citrate buffer (0.01M). Slides were gradually cooled to room temperature and blocked for 30 minutes in 9% goat serum (Zymed, USA) containing 3% Bovine Serum Albumin (BSA, Sigma). Sections were then incubated in primary antibody (see Table I for reference) overnight at 4°C, in a humid chamber. Following washes (3x, 5 minutes), sections were incubated in the respective secondary antibody for 1h at 37°C. After washes (3x, 5 minutes), sections were incubated in ethidium-homodimer 2 (Eth-D2, 1:100; Molecular Probes, Invitrogen, USA), a nuclear dye, for 10 minutes at room temperature. Following two final washes, slides were mounted in Prolong (Molecular Probes, Invitrogen, USA). Control slides were processed identically but primary antibody was omitted.

All antibodies were diluted in Automation buffer (Bi∅media, USA) with 1% BSA, and automation buffer was used for all wash steps.

All secondary antibodies used were from Molecular probes (Invitrogen, USA; Table I).

Follicle morphology and classification

Follicles were classified according to Pederson and Peters (Pedersen and Peters, 1968) and Myers et al. (Myers *et al*., 2004). Briefly, follicles were classified as primordial if the oocytes were partially or completely surrounded by squamous granulosa cells (GCs), equivalent to Type 1-2 in the Pederson and Peters (1968) classification. Primary follicles were those exhibiting one complete layer of cuboidal granulosa cells, (Type 3-3b). In the transition from primordial to primary classification was made according to the predominant type of granulosa cells present. Secondary follicles were classified as all follicles having more than one granulosa cell layer and no visible antrum (Type 4-5). Follicles with a small antrum were designated early antral (Type 6) and antral follicles (Type 7) when the follicle had a single large antral space.

Counting Gap junction foci

Counts were done using the LSM Image Examiner software from Zeiss. Using the fluorescent images collected we've counted all the connexin 37 and 43 foci observed in single plane. The follicles in which the counts were done all had the nuclei in the focal plane to account for double counting.

Image acquisition and analysis

Whole mount or sectioned ovary preparations were examined with a LSM-5 Pascal confocal microscope (Zeiss, Germany) mounted on a Zeiss Axovert 200M microscope equipped with Diode laser (405nm), Argon laser (458, 477, 488 and 514nm) and Helium Neon laser (594nm). Single scans or Z series data sets were made using either 20x, 40x, 63x and 100x objectives (Zeiss, NA=1.25) after the necessary adjustments in gain and offset to minimize saturation. Acquired data files were analyzed using LSM Image Examiner software.

***In situ* Hybridization**

GDF9 sense and antisense dioxigenin-labeled riboprobes were generated from plasmid templates, using a DIG RNA Labelling Kit (SP6/T7 – Roche Diagnostics) in accordance with manufactures instructions. The ovaries for in situ hybridization were in paraffin sections and fixed in 4% paraformaldehyde. Pretreatment, hybridization, and post-hybridization washes were preformed according to protocols described in the Non-radioactive In Situ Hybridization Application Manual (Roche Diagnostics). GDF9 sense and antisense probes were hybridized overnight. Probes were detected immunohistochemically using alkaline phosphatase (Molecular probes) according to the manufacture protocols.

Statistical Analysis

Data are presented as mean ± s.e.m. (Standard Error of the Mean) and statistical analysis of follicle number counts was performed using Prism 4.0

(GraphPad Software Inc., San Diego, CA). Data was analyzed using non-parametric Kruskal-Wallis test, which compares all the groups, and the post-test of Dunn's that compared between ages. In both cases, $P < 0.05$ was considered statistically significant.

RESULTS

Oocyte paracrine regulation of ovarian folliculogenesis

Regarding GDF9 distribution in the C57Bl/6 mouse strain, evaluated through the use of a specific antibody (a gift from Dr. Martin Matzuk's laboratory, Fig. 1), we observed that GDF9 was present at all follicle stages including primordial follicles, and even in germ cell clusters (Fig. 1 D-F). In early adulthood (P20), it was possible to confirm GDF9 expression in the cytoplasm of growing oocytes (Fig. 1C and F, arrows).

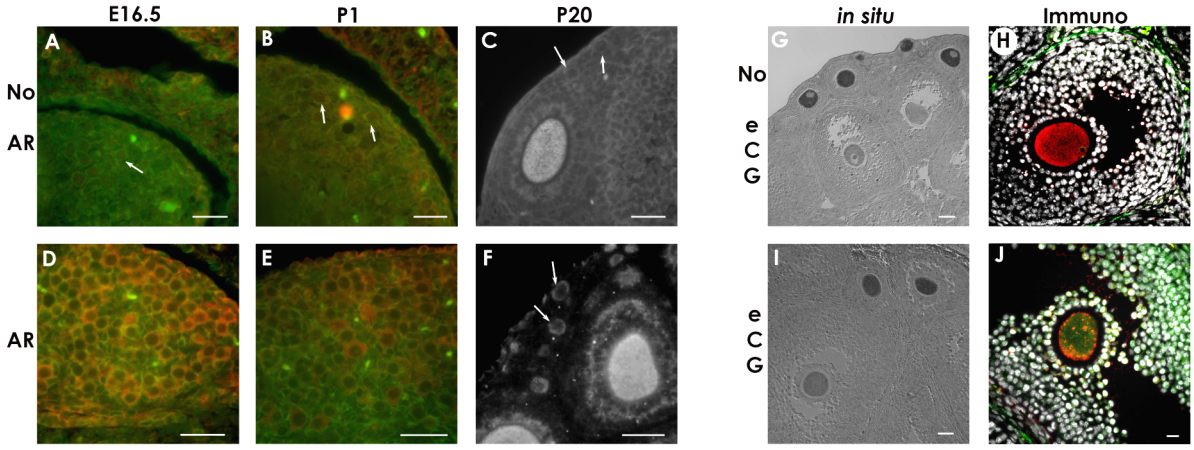

Figure 1 – GDF9 protein and mRNA expression in C57Bl/6 mouse ovaries. **A**-**C** no antigen retrieval (No AR). **D**-**F** antigen retrieval (AR). In **A**-**B** and **D**-**E** GDF9 in red and tubulin in green. **C** and **F** GDF9 in white. **G** and **I** P21 ovaries *in situ* hybridization developed with alkaline phosphatase. **H** and **J** P21 ovaries immunoflourescence GDF9 red, tubulin in green and white nuclei. **G**-**H** unprimed and **I**-**J** primed. White arrows pointing germ cells in A-B and primordial follicles in C and F. Bars = 20µm.

To investigate if FSH influenced GDF9 mRNA and protein expression, using primed and unprimed mice, we performed *in situ* hybridization and immunofluorescence analysis in 21 day-old females mice (Fig. 1G and I). We observed that in general GDF9 expression either at the mRNA or at the protein levels is diminished in bigger antral follicles, in both primed and

unprimed ovaries (Fig. 1G-J). In ovarian sections from primed mice the same decrease in oocyte labeling intensity was visible in big antral follicles (Fig. 1I-J).

Germ-somatic cell interplay: hormonal and oocyte involvement

We analyzed the distribution of two specific gap junction proteins, connexin 43 (cx43) and 37 (cx37), in young and adult females to comprehend FSHβ or GDF9 influence in cell-cell communication. This evaluation was done by immunofluorescence for cx43 protein distribution and counting both connexins (43 and 37) foci in 21day-old control (C57/Bl6) unprimed and primed, GDF9 KO, and FSH KO females. For immunoflurescene we used young females (6-day old) GDF9 KO and adult females (42-day old) in FHSβ KO, to better access the protein effects and distribution, since GDF9 KO has no secondary follicles and FSHβ KO has no antral follicles. Whereas in the counting study we compared primary follicle stage only, due to the fact that GDF9 KO's ovaries do not present secondary follicles, and we have observed a relative stability of mean connexin foci distribution per follicle stage per age in FSHβ KO's ovaries (data not shown). The use of unprimed and primed females allowed for a better evaluation of FSHβ effect upon gap junction distribution. Since we have previously, (Chapter 3) observed heterologous gap junction between germ-granulosa cells, we counted the foci within the oocyte surface and within the corona radiata (first granulosa cell layer).

Connexin 43 protein distribution was similar in both GDF9 and FSHβ models. Control C57 images show cx43 double-labeled for actin to better identify the follicle structures (Fig. 2A and D). In either knockout mice models cx43 was conjugated with tubulin to compare follicles morphology in heterozygous and homozygous individuals (Fig. 2B-C and E-F). GDF9 KO showed more primordial and a few primary follicles, in which the oocyte size is bigger than the heterozygous at 6 days post birth (Fig. 2B-C). Connexin 43 was present in granulosa cells, even in the GDF9 KO (Fig. 2C

arrows). Regarding FSHβ, homozygous ovaries, as expected for this mouse strain, there were no antral follicles in the adult ovaries (Fig. 2F).

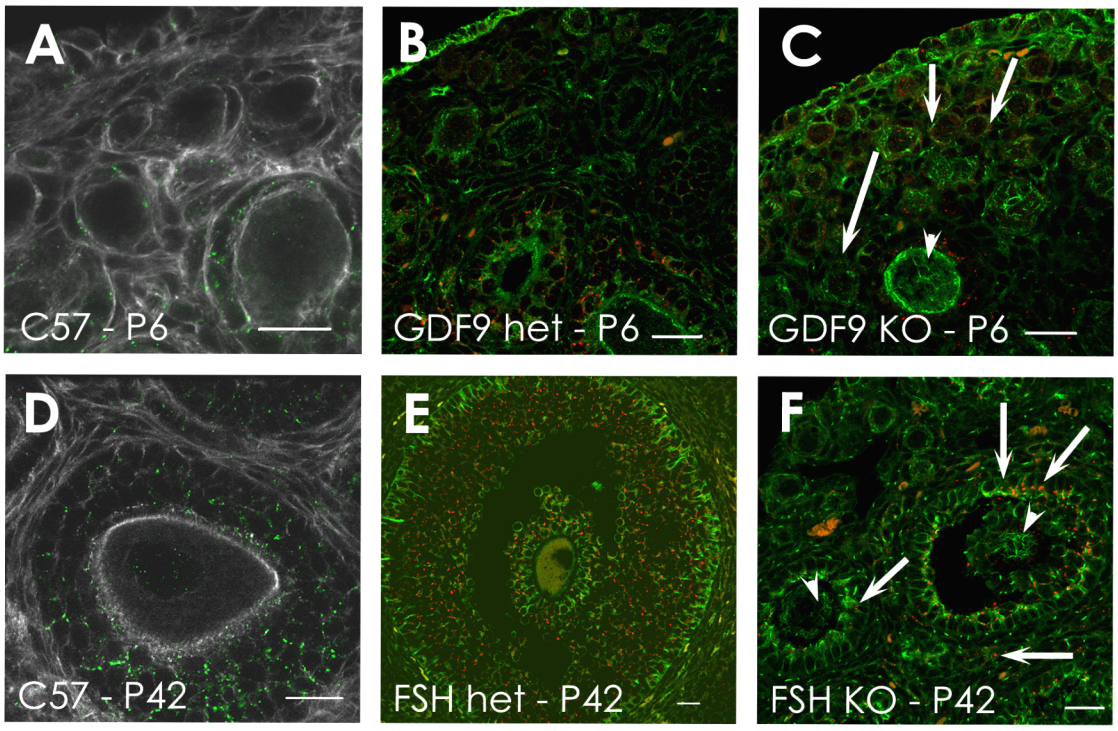

Figure 2 – Connexin 43 cell-cell communication regulation in GDF9 and FSHβ knockout mice model. Control C57 6 days post-birth (P6) (**A**) and 42 days postnatal (P42) (**D**), actin in white and connexin 43 in green; GDF9 P6 tubulin in green and connexin 43 in red, in heterozygous (**B**) and homozygous (**C**) females. FSHβ P42 tubulin in green and connexin 43 in red, in heterozygous (**E**) and homozygous (**F**) females. Bars = 20μm

Connexin 43 was largely present between granulosa cells, the only difference being the lack of antral follicles in FSHβ KO (Fig. 2E-F arrows). This was accompanied in both knockout models by more tubulin bundles in the oocytes than the respective heterozygous (Fig. 2C arrowhead and F arrowheads). Here we used acetylated α-tubulin antibody labeling to monitor post translational alterations that are usually associated with microtubule stability (Piperno *et al.*, 1987). In contrast to control, in both mutant models we observed the granulosa cells that are located closer to the oocyte were more intensely stained than theca cells. We wondered if this could be a result of impaired germ-somatic cell communication of both hormonal and oocyte paracrine defieciency?

To address this question, we have counted connexin foci, in primary follicles. Interestingly, primed ovaries had almost no connexin foci (43 or 37) in primary follicles (Fig. 3).

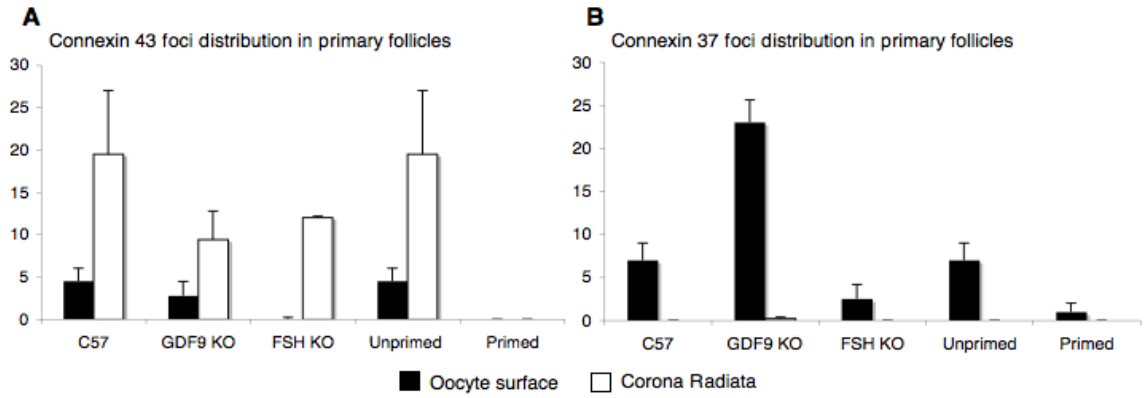

Figure 3 - Gap junction communication in primary follicles in oocyte surface and corona radiata of GDF9 and FSHβ deficient females. A Mean number of connexin 43 foci and **B** Mean number of connexin 37 foci. Black bars Oocyte surface and White bars Corona radiata, in both A and B. Data presented as mean ± s.e.m. (at least 10 follicles per group).

The number of cx43 foci were similar at the oocyte surface – hetereologous gap junctions, in most strains, except for FSH KO and primed (Fig. 3A). The presence of cx43 foci increased between corona radiata, implicitly homologous 43/43 gap junctions, both C57/Bl6 and unprimed had similar gap junction numbers (19.5±7.5). Knockouts, GDF9 (9.4±3.4) and FSHβ (12±1.8), presented cx43 foci numbers in corona radiata very similar between them, however lower than the "controls" (C57 and unprimed) (Fig. 3A).

With cx37 labeling (an oocyte specific gap junction protein), foci were almost exclusively observed at the oocyte surface (Veitch *et al*., 2004; Simon *et al*., 2006). For this connexin, controls, C57 and unprimed, showed a similar number of foci (7±2), while primed (1±1) presented the smallest foci number (Fig. 3B). Interestingly, FSHβ KO (2.5±1.7), presented the second lowest cx37 foci number (Fig. 3B). The primary follicles that showed the highest number of foci were from the GDF9 KO (23±2.6), in accordance with previously published results (Carabatsos *et al*.,1998).

Together these results demonstrate a relationship in germ-somatic cell interplay related to the acetylated α-tubulin cytoskeleton and gap junctions, at least in the two mutant mice models used. To further investigate the influence of the oocyte role during the period of ovarian follicle assembly, we examined two additional mouse knockout models, Nobox and Sohlh1, which are germ cell specific genes.

Oocyte induced morphological changes during follicle assembly

Oocyte involvement in follicular assembly was investigated taking advantage of two oocyte-specific mouse models – Nobox (homeobox gene) and Sohlh1 (transcription factor), both of which have been implicated in early germ cell loss (Rajkovic *et al*., 2004; Pangas *et al*., 2006). To investigate germ cell death we first monitored cytoplasmic and organellar acidification in whole ovaries of Nobox and Sohlh1 deficient mice, by labelling whole overies with Lysotracker Red (LTR – Fig. 4). Both knockout models showed a similar amount of germ cell acidification (size and location) as controls (wild type and C57) (Fig. 4A-E). However, Sohlh1 KO ovaries also have a much greater number of non-germ cells that were acidified (Fig. 4E). We next wondered if these results were related to germ-somatic cells interplay and early germ cell loss?

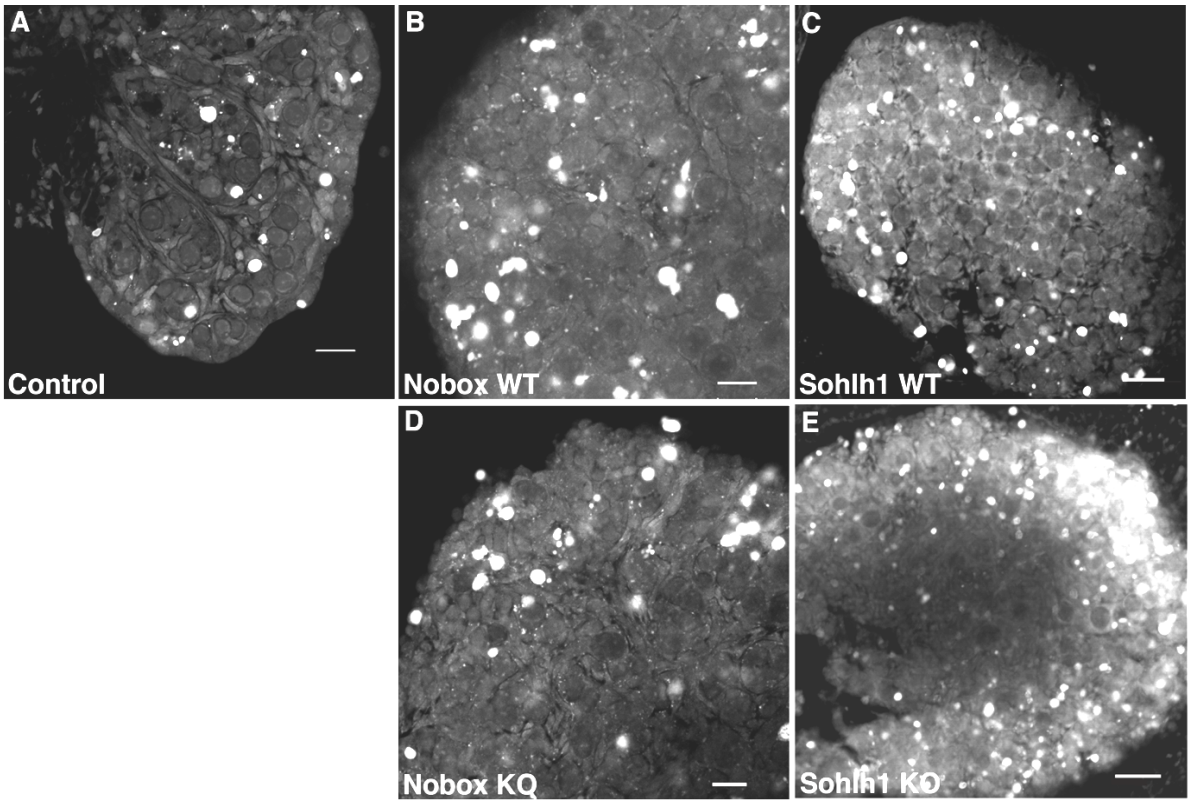

Figure 4 – Cytoplasmic and organellar acidification in ovaries of Nobox and Sohlh1 knockout mice compared with control (C57) and wild type. **A** control C57Bl/6 P2 ovary; **B** Nobox wild type newborn ovary; **C** Sohlh1 wild type P3 ovary; **D** Nobox knockout newborn ovary; and **E** Sohlh1 knockout P3 ovary. Bars = 20µm.

To address this question, we have looked at cytoskeleton microtubules (tubulin), ECM component (HSPG) and cell-cell junction (cadherins) at birth, comparing C57 postnatal day (P6) ovaries with GDF9 KO P8 ovaries and C57 P2 ovaries with newborn ovaries of Nobox and Sohlh1 (Fig. 5). We observed similar tubulin distribution in all mouse strains; microtubules appeared as fin-threads lace-like leading to a cortical band underlining germ and somatic cells in the ovaries (Fig. 5A-E). All knockout models seem to have a looser cytoskeleton structure when compare with C57's control ovaries. GDF9 KO ovaries have follicles like C57, but structurally, the microtubules appeared looser than control (Fig. 5 A and B). In newborn Nobox KO ovaries, tubulin delimited the oogonia clusters (Fig. 5D circle) more intensely but it was also more lace-like around the oogonia, as we reported previously (Chapter 3). Compared with control (Fig. 5C), Nobox KO ovaries seem to have more primordial follicles, as if early cluster

breakdown had already occurred. Newborn Sohlh1 KO ovaries showed an identical tubulin distribution as Nobox KO (Fig. 5E). Clusters were rarely observed.

Regarding HSPG and cadherins, all knockout mouse models had a more disorganized appearance compared to control with respect to ECM – HSPG (Fig. 5F-G) and cell-cell junctions – cadherin (Fig. 5F-O). In Nobox and Sohlh1 KOs, although it seemed that same basal staining was observed, HSPG is much more intense in the ovarian surface (Fig. 5I-J). Regarding cadherins, the control is stained with E-cadherin, and as shown is only present in the extra ovarian epithelium (Fig. 5M). The KO's ovaries were stained with a multi-Cadherins antibody, which recognizes more than E-Cadherin. This is why the pattern of cadherin in Nobox and Sohlh1 KO's seemed so different from control (Fig. 5N-O). In both these mouse models the ovarian surface seemed looser (Fig.5N-O).

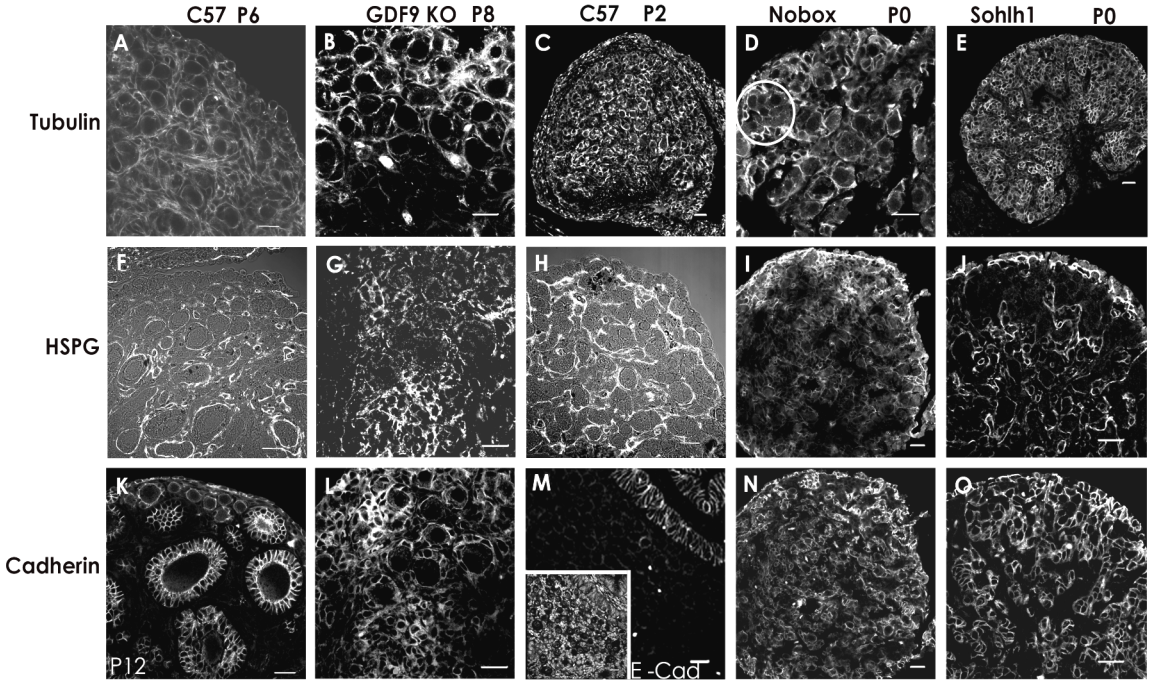

Figure 5 – Cytoskeleton and extracellular matrix proteins in genetically manipulated mice models. A-E acetylated tubulin distribution in white; **F-J** HSPG distribution in white; and **K-O** cadherin distribution in white; in C57Bl/6 ovaries at 6 day post-natal (P6) and P2, except for cadherin distribution P12. (A, F and K – P6; C, H and M – P2 insert in M nuclei and cadherin in white), GDF 9 homozygous P8 ovaries (B, G and L) NOBOX homozygous newborn ovaries (B, E and H), and Sohlh1 homozygous newborn ovaries (C, F and I). Bars=20µm

Oocyte-specific genes have been implicated in the maintenance of female germ cells and here it seems that this equilibrium is related with an interrelation of the ovarian structure and germ-somatic-ECM interplay.

DISCUSSION

Ovarian morphology and functionality has been the subject of a plethora of studies since in 2004 the biological dogma of ovarian follicle renewal in adult mammalian females was challenged (Johnson *et al*., 2004). This challenge despite generating a heated debate among the reproductive scientific community, also led scientists to realize the gap in their knowledge on ovarian histogenesis.

Most of what is known today in mammalian ovarian development results from morphological descriptions of ovaries at different ages in different models, and of targeted disruption of genes in the mouse models. This last tool has enable researchers to identify the regulators of ovarian function and also as models of human disorders affecting reproduction.

In the present study we aimed to enhance the understanding of follicle formation and both oocyte and hormonal involvement upon the shaping of the ovarian follicular reserve. For that purpose we used GDF9, Nobox, Sohlh1 and FSHβ deficient mice models and showed for the first time that GDF9 is present at all stage follicles and when deleted, it modifies oocyte-granulosa-matrix cell communication, as do Nobox and Sohlh1 which act upstream of GDF9. The importance of ovarian cell cytoskeleton and communication (gap and adhesion junctions) is fundamental for normal establishment of the germ cell reserve.

Hormonal control of ovarian follicle reserve

The involvement of FSH in primordial follicle assembly has been suggested to occur in the hamster (Roy and Albee, 2000). At birth it is possible that the acute drop in Estradiol (E_2 – caused by birth and separation from maternal

circulating E2) together with the circulating FSH, cause an up-regulation in Activin A (ActA), thus inducing the expression of germ cell specific factor – *Figla* and *Nobox* (Lei *et al*., 2010), genes that are essential for primordial follicle formation (Soyal *et al*., 2000; Rajkovic *et al*., 2004). Recently, Cossigny and colleagues (2012) suggested that FSH effects the survival of rat in vitro cultured primordial follicles and supports ActA action on primordial to primary follicle transition. FSH contribution to primordial follicle assembly is probably coordinated with local factors independent of this hormone (Lei *et al*., 2010; Durlinger *et al*., 2001). One of these factors may be AMH, which was shown to inhibit not only primordial growth, but also FSH-stimulated follicle growth (Durlinger *et al*., 2001). Interestingly, AMH was also shown to inhibit follicular assembly, acting mostly in the stromal-epithelial cell interaction (Nilsson *et al*., 2011). Interestingly, ActA can also increase primordial follicle number when administered exogenously to neonatal mice (Bristol-Gould *et al*., 2006a). These authors created an empirical mathematical model to study the significant changes induced by ActA upon the ovarian follicular reserve. They suggest that an optimal follicle number exists and if exceeded, oocyte quality declines, implying that the ovary possesses a mechanism to eliminate excess follicles (Bristol-Gould *et al*., 2006b). Cossigny and colleagues (2012) proposed something similar, when they observed a decrease in preantral follicle numbers if mice were treated with ActA and FSH, suggesting that apoptosis may be the means by which the ovary eliminates the poor-quality oocytes.

Activin A was also implicated in somatic cell proliferation, related to peripheral distribution of connexin 43, on granulosa cells, in a bovine pre-antral follicle culture system (McLaughlin *et al*., 2010). These authors proposed that Act A supports folliculogenesis by inducing cell contact interations (McLaughlin *et al*., 2010). Here we have looked at connexin 43 distribution in adult ovaries, and counted foci in primary follicles. Although, we did not observe a peripheral cx43 distribution in mouse ovaries, we did detect increased microtubule stability on the periphery of the antral

follicles of FSHβ heterozygous (control) ovaries, which was absent in FSHβ deficient ovaries. In fact, we detected an inversion of this effect, and the granulosa cells more close to the oocyte in the FSHβ deficient ovarian follicles have more stable microtubules than the FSHβ heterozygous ovaries. This is consistent with a previous study (Combelles *et al*., 2004), where the authors report the retraction of TZP's with the exposure to FSH. Both events, microtubule stability and TZP retraction, may be related to antrum formation and hence impaired communication between somatic and germ cells in the FSHβ deficient females. This fact was reinforced by connexin 37 foci counting results, where the primed and FSHβ deficient females exhibited the lowest numbers of cx37 foci, whereas controls and unprimed females had higher numbers. As shown previously, both connexins have an important role on follicle development (Veitch *et al*., 2004; Teilmann, 2005) and at least connexin 43 is associated with TZP's (Teilmann, 2005; Li and Albertini, 2013- review).

Together these results suggest that the absence of FSHβ unbalances germ-somatic cell communication in later folliculogenesis stages, observed by altered gap junction and TZP patterns.

Oocyte control of ovarian follicle reserve

In this study we took advantage of three mutant oocyte-specific mouse models: GDF9, NOBOX and Sohlh1 knockouts, which all led to female infertility and in two of them - Nobox and Sohlh1 - to premature ovarian failure (complete germ loss at early ages).

GDF9 is an oocyte growth factor essential for primary to secondary follicle transition (Vitt *et al*., 2000). It also promotes primordial follicle progression (Vitt *et al*., 2000). This growth factor induces granulosa cell proliferation (Vitt *et al*., 2000). Hence the null females have no secondary follicle formation, although the oocyte continues growing (Dong *et al*., 1996; Carabatsos *et al*., 1998). Carabatsos and colleagues (1998), reported a "finite capacity"

of granulosa cells proliferation in GDF9 null females, yet nuclear remodelling is consistent with oocyte maturation and growth. This is in agreement with recent findings on theca cell lineage and its requirement for GDF9 action in granulosa cells leading to the correct formation and function of theca cells, and hence normal follicle development (Liu *et al*., 2015).

In the present study we show for the first time GDF9 mRNA and protein expression in primordial mouse follicles. Previously, it has been reported GDF9 mRNA and its protein expression was excluded from the oocyte at all developmental stages except primordial type 2 (McGrath *et al*., 1995; Elvin *et al*., 1999b). Regarding the GDF9 mRNA expression the difference might be related with the detection method used by McGrath and colleagues (McGrath *et al*., 1995), which used a radioactive label detected by autoradiography. However, our study used a non-radioactive digoxigenin labelled probe detected by immonohistochemestry using alkaline phosphatase, a method that is more "sensitive, reliable, and efficient alternative to radio-labeled probes for in situ hybridization of mRNA" (Komminoth *et al*., 1995). Previously, GDF9 mRNA expression in primordial follicles was also reported in ovine and bovine using radioactive probe labeling (Bodensteiner *et al*., 1999). On the other hand, the fact that we antigen retrieved the paraffin slides before primary antibody incubation allowed to detect GDF9 protein expression in primordial follicles, which previously has not been done (Elvin *et al*., 1999b). A difference in techniques may be the reason why, until now, there were no reports of GDF9 in mouse primordial follicles. However, due to the implication of GDF9 in the primordial follicle formation in hamster (Wang and Roy, 2006), we also examined GDF9 KO ovaries. Even though the ovarian structure is severely compromised, primordial and primary follicles are formed, indicating that GDF9 may participate in, but is not essential for primordial follicle formation in mice. Nevertheless, the suggested that FSH regulates GDF9 expression is partularly interesting (Wang and Roy, 2006). We have

looked at pubertal ovaries, to see if either mRNA or protein expression was altered with FSH increase. However, we found no changes which is in accord with previous results that suggested GDF9 as an inducer of granulosa cell proliferation from primary until preovulatory follicle stage, inhibiting FSH-induced steroidogenesis and LH receptors expression, in mice (Vitt *et al*., 2000). This is well illustrated when one compares between GDF9 KO and FSH KO. The FSH KO ovary maintains its follicular growth until preantral stage, whereas GDF9 KO arrests in the primary stage. GDF9 is essential for granulosa cell proliferation which was also confirmed in human follicles (Hreinsson *et al*., 2002). The addition of exogenous GDF9 in *in vitro* matured (IVM) mouse oocytes, together with FSH and EGF (epidermal growth factor) improved embryo development and fetal viability (Yeo *et al*., 2009). Again recently, GDF9 influence on granulosa cells was found essential for *Dhh*/*Ihh* expression in these cells and it's action in theca cell induction (Liu *et al*., 2015). This may reinforce the explanation why GDF9 KO follicles arrest at primary stage.

Regarding cell-cell communication, GDF9 KO ovaries, presented the lowest number of cx43 foci in the corona radiata, or the first layer of granulosa cells demonstrating a decrease in the number of heterologous gap junctions and decreased communication between corona radiata cells and the oocyte. Interestingly, the highest number of cx37 foci on the oocyte surface was observed in GDF9 KO ovaries compared with the other mouse strains examined. This results consistent with oocyte growth and deficient communication between oocyte-granulosa cell described previously (Carabatsos et al. 1998). Other than the lower number of foci in the corona radiata cells of the GDF9 KO follicles, there was no overall difference in Cx43 distribution between GDF9 KO, and control ovaries (GDF9hets or C57), despite the obviously abnormal ovarian morphology of the GDF9 KO.

Similar observations of cx43 distribution together with GDF9 expression in cx43 KO females lead to the believe that the impaired folliculogenesis in cx43 KO mice is due to reduced responsiveness of granulosa cells to oocyte-produced GDF9 (Gittens *et al*., 2005). The authors proposed that the interplay between paracrine signaling and gap junction communication is necessary for normal folliculogenesis to occur (Gittens and Kidder, 2005). Here we have also found a subpopulation of cx43 at the oocyte surface, which had previously been suggested to directly connect gap junctions with TZP's (Carabatsos *et al*., 2000; Albertini *et al*., 2001; Teilmann, 2005).

To further investigate the oocyte involvement in early folliculogenesis, we used two other null mouse models of germ cell specific genes: Nobox and Sohlh1. These models have early folliculogenesis arrest, both form no primary follicles: Nobox KO has no oocytes by postnatal day 14 (Rajkovic *et al*., 2004), and Sohlh1 by postnatal week 3 has no oocytes (Pangas *et al*., 2006). Nobox and Sohlh1 are transcription factors upstream of GDF9. To investigate a possible pathway for early germ cell loss, we used a modified LTR method and confocal microscopy so that intact living ovaries (control C57/Nobox KO/ Sohlh1 KO) could be evaluated by digital image analysis (Fig. 4). Both control and knockout ovaries exhibited similar cytoplasmic acidification of germ cells that were characterized by their size and disposition. Only Sohlh1 KO ovaries exhibited a higher amount of acidified somatic cells. Previously we have shown that early germ loss occurs through several death mechanisms, and apoptosis via the lysosome pathway is one of the most preferred cell death mechanisms (Rodrigues *et al*., 2009). Here we have seen that this pathway is not preferentially used in the early germ cell loss of these mouse models, however in the absence of Sohlh1 gene, it seems to be the preferential cell death pathway of somatic cells. Given the recent findings on granulosa cells precursor and specification, (Mork *et al*., 2012; Hummitzsch *et al*., 2013), these Sohlh1 results, may be consistent with incorrect granulosa cell specification,

leading to deficient germ cell formation and hence early loss.

To better evaluate ovarian structure we examined cytoskeleton (tubulin), ECM (HSPG) and cell junction connections (cadherins) in ovaries of young GDF9 KO females, and newborn Nobox and Sohlh1 deficient mouse models. For all markers tested, the patterns were identical to control (C57), revealing the phenotypic features that characterize each mutant ovary. For example, HSPG was basally expressed in the ovarian surface epithelium and in surrounding the cells involving clusters, whereas in both mutant models it was not as evident, probably due to the rarity of the clusters. Also the surface epithelium seemed loose, facilitating oocyte shedding during cluster breakdown, as seen previously (Rodrigues *et al*., 2009).

Nobox directly regulates the transcription of *Gdf9* and *Oct4* genes in oocytes, through Nobox Binding Elements (NBEs) (Choi and Rajkovic, 2006). Oocytes from mice lacking Nobox have several germ-cell specific genes down regulated, including *Gdf9* (Rajkovic *et al*., 2004). Recently, it was suggested that the early oocyte loss in Nobox KO mice is a result of impaired signaling between somatic and germ cell during embryonic development (Lechowska *et al*., 2011). This suggestion is consistent with our observations of impaired communication between oocyte and granulosa cells in the GDF9 ovaries. The fact that Sohlh1 up-regulates Nobox (Pangas *et al*., 2006), which in turn regulates GDF9 (Choi and Rajkovic, 2006), led us to suggest an impaired germ-somatic cell communication for Nobox and Sohlh1 null mice.

Our results demonstrate that a deficient germ-somatic-matrix communication can account for at least part of the abnormalities observed in ovarian development and underscores that even at the early stages of ovarian differentiation reduced or lost fertility will be an unavoidable outcome of such disturbances.

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Table I – Primary and secondary antibodies combinations in paraffin section immunofluorescence.

Primary Antibody	Secondary Antibody	Observations
Acetylated-Tubulin (1:100, Sigma)	Alexa-fluor 488 (1:800, Molecular Probes)	Tubulin marker; fixed in PFA and antigen retrieved.
Heparin Sulphate ProteoGlycan – HSPG (1:100)	Alexa-fluor 488 (1:800, Molecular Probes)	Extra cellular matrix marker done in paraffin sections fixed in PFA, antigen retrieved.
Pan-Cadherin (1:500, Sigma)	Alexa-fluor 488 (1:800, Molecular Probes)	Multi-cadherin marker (E-Cadherin, N-Cadherin (A-CAM), P-Cadherin, V-Cadherin, R-Cadherin and T-Cadherin); paraffin sections fixed in PFA with antigen retrieved.
Connexin 43 (1:100; Santa Cruz)	Alexa-fluor 488 / 568 (1:800, Molecular Probes)	Gap-junctional marker; paraffin sections fixed in PFA, antigen retrieved.
GDF9 (1:120; gift from Dr. Martin Matzuk Lab)	Alexa-fluor 568 (1:800; Molecular Probes)	GDF9 protein marker specific of germ cells cytoplasm; with and without antigen retrieval.

Dyes	Alexa-fluor 568 F-Actin (1:200, Molecular Probes), dye used for staining actin intermediate filaments in red. Hoechst (1 µg/ml; Polysciences, Inc.), dye used for staining DNA in blue. Eth-D2(1:100; Molecular Probes), dye used for staining DNA in red.

CHAPTER # 5

CONCLUDING REMARKS

The ovary is a remarkable organ on which determines a female's reproductive lifespan based upon an equilibrium between the survival and death of follicles. This equilibrium, in the ovary, is initiated with its formation and consequent assembly of oocyte-containing follicles (Pepling and Spradling, 2001; Rodrigues *et al*., 2009) and is maintained by their cyclical growth and ovulation (McGee and Hsueh, 2000).

Two subsets of ovarian follicle reserve have been proposed, which are themselves related with these two equilibrium periods (Monniaux *et al*., 2014). These subsets are the primordial follicle reserve established early in ovarian development, and the second population constituted of follicles responsive to gonadotropin, the dynamic reserve for ovulation (Monniaux *et al*., 2014). The two reserves are related with one another at all levels, including genetic and environmental (Monniaux *et al*., 2014).

Hirshfield (1992) first proposed the two primordial follicle population hypothesis over two decades ago. The medullary primordial follicles start growing as soon as they are formed and the cortical primordial follicles mature gradually over the reproductive life span (Hirshfield, 1992; Zheng *et al*., 2014b). Only recently however, has strong support her hypothesis started to emerge.

Mork and colleagues (2012), elegantly showed, combining tamoxifen-inducible labelling of *Foxl2*-expressing cells with BrDU/MitoTracker-based cell lineage tracing, that the supporting cells of the primordial follicles in the ovarian medulla arise from the surface epithelium of foetal ovary, whereas the supporting cells of the primordial follicles in the adult ovary cortex arise from the surface epithelium of the post-natal ovary (Mork *et al*., 2012).

Similarly, Zheng and co-workers (2014a), using tamoxifen-inducible reporter mouse model, *Foxl2-CreER^{T2} and Sohlh1-CreER^{T2}*, labelled the primordial follicles activated in the ovarian medulla and cortex, respectively. This work showed that the first wave of primordial follicles are activated simultaneously, and fast growing, have a key role at the onset of puberty, and provide the oocytes until 3 month of age (Zheng *et al*., 2014a). Whereas the second wave is comprised of primordial follicles derived from the ovarian cortex, is slow growing, and this population contributes to the continuous ovulation throughout the adult reproductive lifespan (Zheng *et al*., 2014a). Interestingly, these results are also in agreement with a new hypothesis of development of the mammalian ovary and follicles (Hummitzsch *et al*., 2013). This hypothesis describes a model whereby mesonephros-derived somatic cells contribute to the formation of ovaries and follicles. Both ovarian surface epithelial and granulosa cells have a common precursor cell – GREL – Gonadal Ridge Epithelial-Like (Hummitzsch *et al*., 2013).

In addition, it was recently shown that theca cells arise from two different progenitor cells: *Wt1*-positive cells from foetal ovary and *Gli1*-positive cells from mesonephros (Liu *et al*., 2015). *Gli1* theca cell marker is expressed in response to signals from oocyte (GDF9) to the granulosa cells (Dhh/Ihh) (Liu *et al*., 2015).

Collectively, these reports clearly illustrate how well coordinated the ovary needs to be in order to produce a quality oocyte for a healthy baby to be born.

Our research focused on the multicellular ovarian cells structure to better understand the establishment and maintenance of the ovarian follicle reserve.

We first studied foetal and neonatal germ cell loss, finding multiple mechanisms involved. We then observed somatic-germ cell interplay and its influence in the ovarian follicle reserve, and found evidence for the two follicle populations concordant with the newer ovarian development

hypothesis. Next we investigated the oocyte and hormonal influence at the somatic-germ cell interface, using several convenient knockout mouse models, showing the fundamental importance of this cell-cell communication.

Germ cell loss mechanisms

We showed that multiple mechanisms contribute to early germ cell loss at the time of follicle formation, and consequent ovarian follicle reserve. Besides apoptosis, we found that germ cell shedding and autophagy are two other important, non-classical, mechanisms involved in germ cell loss.

Tigen and colleagues (2009) also showed that in pubertal mouse primordial follicle loss occurs through non-classical apoptosis pathway. These authors did not investigate the authophagy pathway. Concomitant with our study Escobar and colleagues (Escobar *et al*., 2008), concluded that a combination of apoptosis and autophagy is responsible for germ cell elimination in newborn rats. Interestingly, a recent study proposed autophagy as an important germ cell survival mechanism for the "germ cell endowment" prior to follicle formation (Gawriluk *et al*., 2011). The authors compared the germ cell numbers in Beclin-1, mammalian protein involved in enucleation and autophagosome maturation phases of autophagy, heterozygous females and Atg7, protein involved in vesicle elongation phase of autophagy, homozygous female (Gawriluk *et al*., 2011). Beclin-1 exhibited 56% less germ cells than the wild type and Atg7 had no discernible germ cells to evaluate (Gawriluk *et al*., 2011). Recently, the absence of Atg7 induced germ cell over-loss in neonatal ovaries (Song *et al*., 2015). The authors show, using the same autophagy and caspase (apoptosis) inhibitors as we did in our study – 3-MA and Z-VAD, respectively, that under starvation conditions, the autophagic gene stops massive germ cell loss by apoptosis (Song *et al*., 2015).

This is in agreement with the proposal that an optimal number of oocytes are present in the mouse ovary, at puberty (Bristol-Gould and Woodruff,

2006). According to this proposal, the surplus oocytes would be eliminated and would coincide with poor quality oocyte (Bristol-Gould *et al*., 2006). In fact, it was suggested that depending in which contest the germ cell finds itself, it has the ability to activate different cell death pathways possibly to eliminate poor quality oocytes (Lobascio *et al*., 2007). Similarly, in older female mice, it was shown that eEF2K – elongation factor 2 kinase, regulates germline quality and elimination of defective oocytes (Chu *et al*., 2014). Knocking down eEF2K reduces apoptosis, increasing oocyte survival, however at the expense of accumulating defective oocytes (Chu *et al*., 2014). Again demonstrating the importance of a death-survival equilibrium within the ovary.

Recently, a pro-apoptotic gene BBC3 (BCL2-binding component 3, also known as PUMA), BH3-only protein belonging to the BCL2 family, was shown to regulate the number of embryonic germ cells before meiotic entry (Myers *et al*., 2014). Its absence translates in an increased number of primordial follicles, hence increasing the follicular reserve (Myers *et al*., 2014). Another survival pathway was shown to be active in ovarian follicles, the hippo signalling pathway (Sun *et al*., 2015), in particular a key member of this pathway – Last1, a kinase, necessary for germ cells maintaining (Sun *et al*., 2015).

These studies are evidence of multiple mechanisms/pathways for ovarian germ cell reserve establishment, and at the same time of the remarkable complexity of the ovary.

Ovarian follicle reserve: somatic-germ cell interplay

In this study we show the importance of matrix-somatic-germ cell interactions in establishing the ovarian follicle reserve in the perinatal mouse ovary. The Notch pathway seems to be involved and, together with gap junctions, may be determining which follicles will activate immediately after formation in the medulla of the ovary, hence belonging to the first primordial follicle population, which are destined for elimination.

Our results are consistent with the hypothesis of two primordial follicle classes ((Hirshfield, 1992; Mork *et al*., 2012; Zheng *et al*., 2014a; Liu *et al*., 2015; reviewed: Monniaux *et al*., 2014; Zheng *et al*., 2014b)). And, our finding emphasize the importance of somatic-germ cell bidirectional communication as it has been reported over the past decades (Eppig *et al*., 1997; Albertini *et al*., 2001; Eppig, 2001; Matzuk *et al*., 2002; Rodrigues *et al*., 2008; Li and Albertini, 2013).

As mentioned above, in 2013 Hummitzsch and colleagues proposed a new model for ovary and follicle development, which instead of the surface epithelial cells penetrate into the ovary to form the granulosa cells, both epithelial and granulosa cells are believed to derive from the same precursor cell (Hummitzsch *et al*., 2013). These precursor cells proliferate into the gonadal ridge along with the primordial germ cells and with cluster breakdown, they differentiate into granulosa cells (Hummitzsch *et al*., 2013). Our results are in agreement with this new model, in particular regarding the ECM and ovarian surface epithelium observations. The presence of heterologous gap junctions in the ovary, since foetal ages, also seems to be in agreement with the new ovarian development hypothesis.

Our work continues to demonstrate the complexity of the ovary with the involvement and coordination of multiple pathways to ovarian ontogeny and follicle reserve establishment.

Somatic-germ cell interplay: lessons from knockouts

Targeted gene disruption – knockouts, have become a powerful scientific tool, helping investigators to comprehend better the function of a specific gene by silencing it (Roy and Matzuk, 2006). We took advantage of this tool and used three mouse models where germ cell specific genes were silenced – GDF9, Nobox and Sohlh1, and another where a hormone, pivotal for follicle growth was deleted - FSHβ. We summarize in figure 1 the main function of these genes in folliculogenesis, when silenced (Fig. 1).

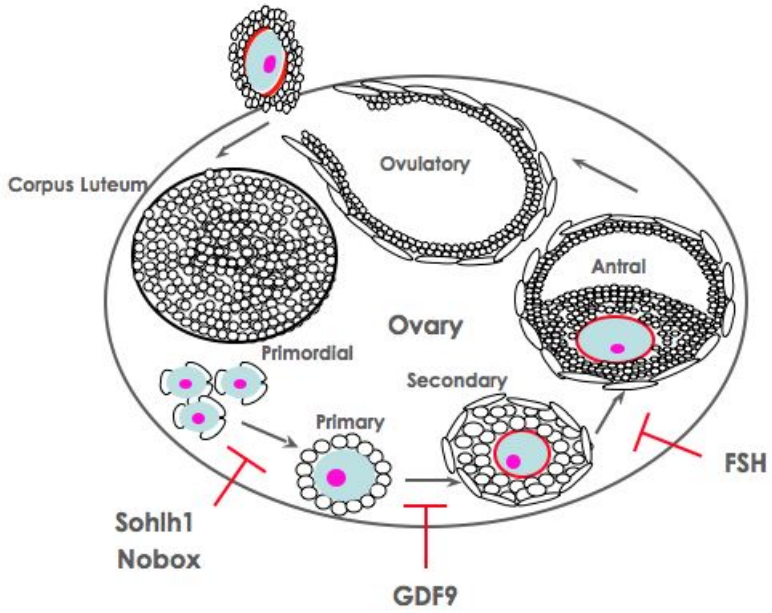

Figure 1 – Schematic representation of main action of particular genes deletion (Sohlh1, Nobox, GDF9 and FSHβ).

Our findings reinforced the importance of somatic-germ cell interplay in ovarian follicle establishment and development. All of these genes when silenced induce female infertility in mice (Fig. 1 and 2). Each gene has its own specificity, but generally the silencing of these genes may be summed up as a problem of cell-cell communication. For example, in Sohlh1KO ovaries, the lysosomal apoptosis of somatic cells seem to be responsible for defective and reduced primordial follicle reserve. Or, like in GDF9KO ovaries, where in its absence, besides no granulosa cells proliferation, Dhh/Ihh is not produced and no theca cells are formed, hence follicle growth becomes arrested at the primordial follicle stage. Figure 2 summarizes our main observations relative to somatic-germ cell interplay in the mice models studied.

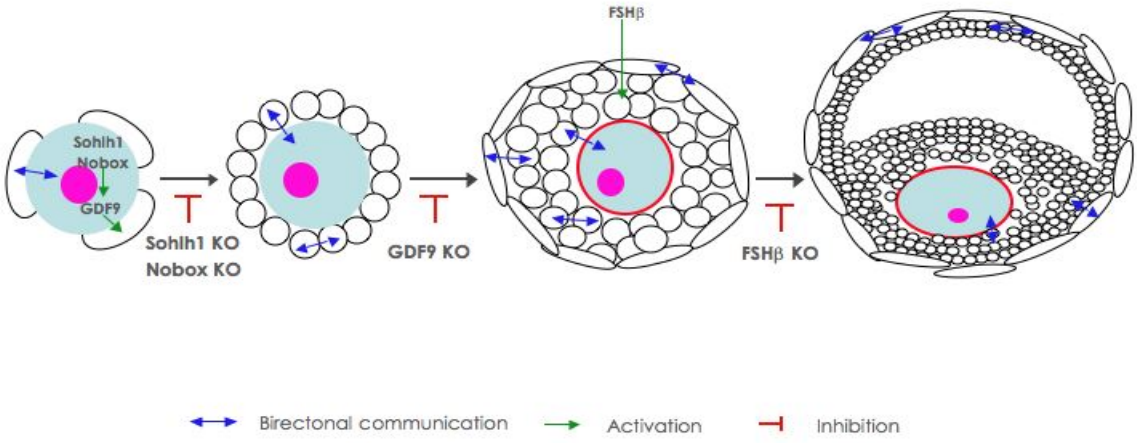

Figure 2 – Schematic summary of results observed in our study.

In humans, these genes (Sohlh1, Nobox and GDF9) are directly or indirectly associated with primary ovarian insufficiency (Bouilly *et al*., 2011; Norling *et al*., 2014; Bouilly *et al*., 2015). Understanding ovary ontogeny and ovarian follicular reserve in the mouse model may allow to better recognize and correct human folliculogenesis defects.

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PORTUGUESE EXTENDED ABSTRACT

Resumo

O ovário é um órgão notável que determina o potencial reprodutivo feminino, com base num delicado equilíbrio entre proliferação, sobrevivência e morte celulares. Neste órgão, este equilíbrio inicia-se com a formação dos folículos ováricos (Pepling and Spradling, 2001; Rodrigues *et al*., 2009) e terá de ser assegurado em conjunto com o crescimento e a maturação foliculares, bem como com a ocorrência de ovulações periódicas (McGee and Hsueh, 2000).

Recentemente, foi proposta a existência de duas sub-reservas ováricas foliculares, as quais aumentam a complexidade deste delicado equilíbrio celular (Monniaux *et al*., 2014). A primeira sub-reserva é constituída por folículos primordiais formados pouco depois da formação do ovário, no inicio do desenvolvimento embrionário. A segunda sub-reserva, é formada por folículos que são receptivos às gonadotrofinas, designada por reserva dinâmica (Monniaux *et al*., 2014). Estas duas sub-reservas estão interligadas, uma não existe sem a outra, exemplificando a importância e sensibilidade deste equilíbrio na regulação e manutenção do ovário e consequentemente da reserva folicular ovárica.

Neste estudo tentámos compreender, com maior detalhe estrutural, como se estabelece a reserva ovárica no modelo experimental do murganho, investigando como a perda inicial de oócitos e a comunicação oócito-granulosa influenciam aquele processo. Iniciámos por contar todas as células germinais presentes nos ovários de murganho em várias idades durante a sua vida fetal (15,5 e 19,5 dias após a fertilização) e após o seu nascimento (2, 6, 10, 12, 20 e 42 dias pós-parto). A contagem e observação de células germinais naquelas fases permitiram confirmar uma redução significativa de cerca de 44% no número total de células germinais após o nascimento. Contudo, a contagem de células germinais apoptóticas não permitiu justificar tal redução apenas com base no mecanismo da apoptose, pelo que foram investigados mecanismos celulares adicionais que permitissem explicar tal redução. Através da

utilização de uma coloração vital nuclear em cultura de ovários fetais, verificámos que ocorre uma perda de células germinais vivas, pela superfície do ovário, coincidente com a altura do nascimento. Por outro lado, a incubação de ovários fetais com um marcador vital sensível ao pH, permitiu observar que existe paralelamente um aumento do compartimento lisossómico nas células germinais coincidente com a altura do nascimento, sugerindo ainda um mecanismo adicional de morte celular. Para melhor compreender este mecanismo efectuámos a cultura de ovários fetais com inibidores específicos de apoptose e de autofagia. Verificámos que os inibidores de autofagia, mas não os inibidores de apoptose, eram responsáveis por reduzir o número de células germinais acidificadas, coincidente com a altura após o nascimento. Os nossos resultados estão de acordo com um estudo recente, onde foram utilizados os mesmos inibidores e em que o gene autofagico, *atg7*, impede a perda massiva de células germinais por apoptose numa situação de escassez de nutrientes, similar ao que acontece logo após o nascimento (Song *et al*., 2015).

Os nossos resultados mostram assim, que vários mecanismos, incluindo a perda de células germinais vivas pela superfície do ovário, a apoptose e a autofagia, estão envolvidos no estabelecimento do conjunto de folículos presentes no ovário a que denominamos de reserva ovárica.

De seguida, investigámos o papel das interacções oócito-pré-granulosa durante a formação da reserva folicular em ovários fetais (19.5 dias após a fertilização), e oócito-granulosa após o nascimento (2, 6 e 20 dias pós-parto). Para isso foram empregues diferentes técnicas de imunofluorescência sobre ovários inteiros ou sobre cortes de parafina, utilizando marcadores para o citoesqueleto (actina, tubulina acetilada, citoqueratina), para a matriz extracelular (ECM – heparano sulfato proteoglicano – HSPG), e para junções celulares (aderentes - β-catenina; de hiato – conexinas 37 e 43). Através da utilização de marcadores de ECM e de junções celulares foi possível demonstrar que até ao dia 2 após

o nascimento o epitélio ovárioco não está ainda completamente formado. A utilização do marcador epitelial mais clássico (citoqueratinas), confirmou que a integridade do epitélio ovárico surge apenas ao dia 20 após o nascimento. Relativamente às junções de hiato, verificámos a sua presença nos folículos ováricos desde o momento da sua formação, e em todas as idades estudadas, sugerindo uma importância constante na formação e manutenção foliculares. Observámos adicionalmente a marcação de Notch 2 em associação com o citoesqueleto (tubulina acetilada) ao nível da interface oócito-granulosa. Fica por confirmar o envolvimento da sinalização Notch nos processos de formação e maturação foliculares, bem como a sua eventual associação à estrutura do cílio primário.

Os nossos resultados parecem estar de acordo com um modelo recentemente proposto para a formação dos folículos ováricos (Hummitzsch *et al*., 2013). Neste modelo, os autores propõem que as células epiteliais na superfície do ovário e as células da granulosa têm uma célula percursora comum. As nossas observações com os marcadores da ECM e do epitélio da superfície ovárica parecem estar de acordo com este novo modelo.

Em conjunto, os nossos resultados apontam para o envolvimento central da interacção entre vários tipos celulares ováricos, germinal e somático, e a matriz extracelular, no estabelecimento da reserva folicular ovárica.

Finalmente investigámos o papel relativo de factores oócitários e hormonais nas interacções celulares nível folicular durante a formação da reserva folicular ovárica. Para isso utilizámos murganhos nos quais genes específicos foram eleminados: GDF9 (growth differentiation factor 9), Nobox (newborn ovary homeobox gene), Sohlh1 (sperma-and oogenesis basic helix-loop-helix1), e FSHβ (follicle stimulating hormone). Estes genes têm sido associados à falência ovárica prematura e a defeitos no desenvolvimento folicular. Concretamente, efectuámos imunofluorescência com os marcadores anteriormente descritos, em

ovários inteiros ou em cortes de parafina nestes modelos de murganho sem um gene especifico. Avaliámos ainda, a distribuição do RNAm do GDF9, por hibridação *in situ*, e da proteína GDF9, por marcação fluorescente, em cortes de parafina. Para verificação dos mecanismos de morte celular, monitorizámos a acidificação celular em ovários de murganhos recém-nascidos Nobox e Solhl1. Ao nível da comunicação oócito-granulosa nos modelos FSHβ e GDF9, observámos deficiências na comunicação oócito-granulosa, as quais foram acompanhadas por alterações espacio-temporais do citoesqueleto, da ECM e das junções aderentes. Todos os modelos utilizados mostraram uma desorganização epitelial e do estroma ovárico, quando comparados com o controlo (estirpes de murganho C57/Bl6 ou CD-1). Verificámos que quer o RNAm quer a proteína de GDF9 se distribuem uniformemente por todas as classes de folículos, incluindo primordiais, ao contrário do que anteriormente tinha sido descrito para murganho (Elvin *et al*., 1999a; Elvin *et al*., 1999b). Relativamente à morte celular nos ovários de Nobox e Sohlh1, ambos mostraram sinais de apoptose e autofagia nas células germinais semelhantes aos animais controlo. Contudo, ao nível das células somáticas, o ovário de Sohlh1 mostrou uma perda celular muito superior, indicando que a ausência deste gene poderá ter um efeito ao nível das células somáticas, e eventualmente relacionado com uma deficiente comunicação na interface oócito-granulosa. Este mecanismo poderá ser uma das causas da falência ovárica prematura, característica deste modelo de murganho.

Mostrámos assim que, tanto factores intrínsecos ao oócito (GDF9, Nobox, Sohlh1), como hormonais extrínsecos ao ovário (FSFβ), podem induzir alterações epiteliais e do estroma ovárico, associadas a anomalias na formação e desenvolvimento foliculares.

Colectivamente, os nossos resultados ilustram a importância das interacções entre o oocito e as células somáticas, e da sua coordenação harmoniosa por forma a que seja produzido um oócito de qualidade, com

competência para assegurar o desenvolvimento embrionário e fetal, possibilitando, em caso de fertilização e implantação, o nascimento de um bebé saudável.

Palavras Chave: reserva ovárica; oócito; folículo primordial; comunicação oócito-granulosa

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ANNEXES

Journal of Cellular Physiology

Oogenesis: Prospects and Challenges for the Future

P. RODRIGUES,[1,3] D. LIMBACK,[1] L.K. MCGINNIS,[1] C.E. PLANCHA,[3] AND D.F. ALBERTINI[1,2]*

[1]*Department Molecular and Integrative Physiology, University of Kansas Medical Center, Kansas City, Kansas*

[2]*Marine Biological Laboratories, Woods Hole, Massachusetts*

[3]*Unidade de Biologia da Reprodução, Instituto de Medicina Molecular, Faculdade de Medicina da Universidade de Lisboa, Lisbon, Portugal*

Oogenesis serves a singular role in the reproductive success of plants and animals. Of their remarkable differentiation pathway what stands out is the ability of oocytes to transform from a single cell into the totipotent lineages that seed the early embryo. As our understanding that commonalities between diverse organisms at the genetic, cellular and molecular levels are conserved to achieve successful reproduction, the notion that embryogenesis presupposes oogenesis has entered the day-to-day parlance of regenerative medicine and stem cell biology. With emphasis on the mammalian oocyte, this review will cover (1) current concepts regarding the birth, survival and growth of oocytes that depends on complex patterns of cell communication between germ line and soma, (2) the notion of "maternal inheritance" from a genetic and epigenetic perspective, and (3) the relative value of model systems with reference to current clinical and biotechnology applications.

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Oogenesis is a protracted process that encompasses the birth, growth, and maturation of a cell unique in its ability to propagate another generation of organisms. In some sense, it is not a very efficient process when measured in terms of viable offspring. In fact, the relative fecundity of a particular organism varies widely according to the kind of reproductive strategy employed. For example, broadcast spawners like fish and many invertebrates are efficient at oogenesis but the fate of ovulated eggs is left up to the whims of the environment the resultant embryos find themselves in. These kinds of animals make a significant metabolic investment in oogenesis. On the other hand, primates expand oocyte numbers prior to birth and engage in a dramatic course of prolonged attrition with a small fraction of the egg endowment surviving to ovulation throughout the reproductive lifespan. As extreme as organisms may be in both the efficiency of egg production and the size of their "spawn" (hundreds to thousands for invertebrates; one for humans), the goals of oogenesis remain the same: producing a developmentally competent egg capable of generating live offspring.

While developmental biologists have long been fascinated by the mechanisms by which an oocyte acquires and executes its totipotentiality during embryogenesis, a new generation of experimentalists have joined the campaign bringing with them research goals of great global and clinical importance. The emergence of the field of assisted reproductive technologies (ARTs) is dominant amongst these new areas of oocyte biology because of the increasing usage of ARTs to treat human infertility. Since the advent of this technology in the late 1970s, more than 3 million children have been born using ARTs and the prospects for innovations and other modifications in current applications are clear. Oocyte and ovary cryopreservation efforts are ongoing as a means to preserve or restore reproductive function to women who have undergone medical treatments that cause partial or complete sterility. Technology for preservation of oocytes, also known as egg banking, is also being viewed as a way to maintain the diversity of living organisms that are rare, endangered, or bred for agricultural or medicinal value. The key point here is that oogenesis is a gradual process by which the properties needed to express developmental competence are acquired at different stages of differentiation and so the timing for intervention strategies and their impact on oocyte quality represent key challenges for the future. In this sense, virtually all clinical applications aimed at female germ line preservation will require identification of appropriate model systems and to unravel the mechanisms that confer and mediate the expression of oocyte developmental competencies.

With this background, the goals of this review are to (1) highlight current mechanistic viewpoints as related to the stages during which mammalian oocytes are born, grow, and mature, (2) revisit the seasoned concept of "maternal inheritance" in light of new data showing that especially in mammals, the relative contributions of the oocytes' endowment to embryo survival and fetal development have been underestimated, and (3) given the impact that studies of oogenesis will have on biotechnology, stem cell research and animal fecundity, the utility of specific animal models will be discussed in the context of future challenges relevant to human health.

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*Correspondence to: D.F. Albertini, Hall Professor of Molecular Medicine, Department of Molecular & Integrative Physiology, University of Kansas Medical Center, 3901 Rainbow Boulevard, Kansas City, Kansas. E-mail: dalbertini@kumc.edu

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Oogenesis is a Protracted Process that Invokes Feedback Regulation at Many Levels

Primordial to primary follicle transition

Immediately after primordial follicle assembly some are recruited from the resting pool into the growing population. The mechanism by which primordial follicles are maintained or leave the resting pool is still not well understood. Primordial follicle activation is progressive and initiated with granulosa cell proliferation, and their change in shape from squamous to cuboidal (Hirshfield, 1991; Braw-Tal, 2002). When these cuboidal granulosa cells form a layer that completely surrounds the also enlarging oocyte, the primordial follicle has become a primary follicle (Fig. 1A–C) (Hirshfield, 1991; Braw-Tal, 2002). Several factors are involved in inhibiting and inducing this transition (Skinner, 2005).

Anti-Müllerian hormone (AMH), the growth factor that leads to Müllerian duct regression in males, has been sited as responsible for maintaining primordial follicles in the resting pool in females (Durlinger et al., 1999, 2002). The observation of early age depletion of primordial follicles in AMH-deficient mice and the fact that AMH is produced in the granulosa cells of developing follicles has lead to the suggestion of AMH as an inhibitor of primordial formation (Durlinger et al., 1999, 2002). To further understand the regulatory action of AMH, Nilsson et al. (2007) performed a transcriptome analysis of ovaries treated with AMH and known stimulatory factors. Results showed that AMH may decrease the expression of pro-activation factors and increase the expression of inhibitory factors (Nilsson et al., 2007). Forkhead transcription factor O3 (Foxo3; FOXO subfamily of forkhead transcription factors which are all downstream effectors of the PTEN/PI3K/AKT pathway) was also implicated in inhibition of primordial follicle activation (Castrillon et al., 2003). These scientists generated mice with a null mutation on the Foxo3 *locus*, and, despite being fairly normal, females exhibited a distinct phenotype of mass follicle activation, leading to early follicular depletion (Castrillon et al., 2003). In fact, a detailed characterization of the Foxo3-deficient ovary showed that Foxo3 is necessary to suppress primordial follicle activation (John et al., 2007); these animals have viable litters as long as there are follicles available but total follicle depletion happens around 15 weeks of age (John et al., 2007). Accordingly, mice where constitutively active Foxo3a was being expressed in the oocytes exhibited infertile females due to slow follicle development and oocyte growth (John et al., 2007). Concomitantly, deletion of PTEN (phosphatase and tensin homolog deleted on chromosome 10; upstream of the Foxo3) gene in mouse oocytes result in activation of the majority of primordial follicles by postnatal day 23, and complete loss by 16 weeks post-birth (Reddy et al., 2008). PTEN is a lipid phosphatase that negatively regulates the phosphatidylinositol 3-kinase (PI3K) signaling pathway of cell proliferation and survival (Cantley and Neel, 1999). It is involved in regulation of primordial follicle activation, since it is this pathway (PI3K–AKT–Foxo3) that kit ligand [KL, growth factor that binds to its cognate receptor – c-kit (Hutt et al., 2006b)] uses to induce oocyte activation (Liu et al., 2006; Reddy et al., 2005). Another signaling pathway, the chemokine SDF-1 and its receptor CXCR4, has been suggested to negatively regulate primordial follicle activation (Holt et al., 2006). Interestingly, in vitro addition of recombinant SDF-1 to neonatal ovaries increased the follicle density but decreased the number of growing follicles when compared to controls, indicating a possible role of this pathway in maintaining primordial follicles in the resting pool (Holt et al., 2006).

On the other hand, KL produced by granulosa cells, is involved in primordial follicle activation (Parrott and Skinner, 1999). Ovaries cultured with and without recombinant KL and/

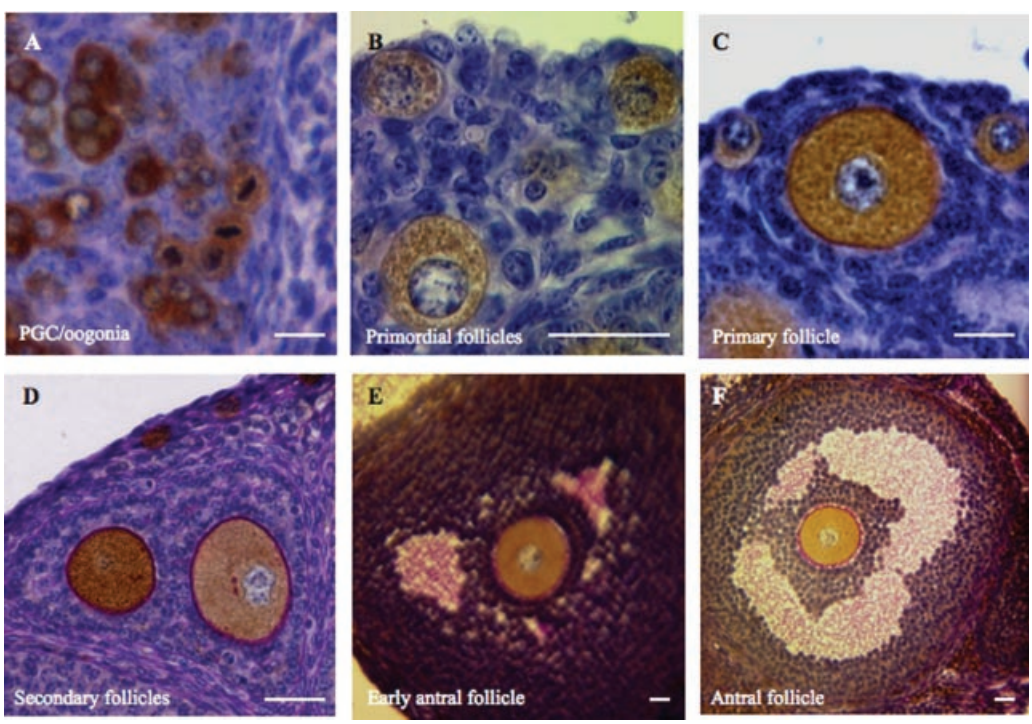

Fig. 1. A–F: Examples of stages in follicle and oocyte growth from oogonia (A) to antral follicle (F). Oocyte cytoplasm is labeled with mouse vasa homolog (MVH) antibody, a specific oocyte factor; zona pellucida is stained with PAS reaction (pink). Note in C the onset of zona pellucida formation in the primary follicle. Scale bars 20 μm.

or neutralizing c-kit antibody (ACK-2) demonstrated that KL is necessary and sufficient to promote primordial follicle recruitment into the growing pool (Parrott and Skinner, 1999). Previously, it had been shown that c-kit (produced by the oocyte) and KL are required in ovarian follicle development prior to gonadotropin dependence (Yoshida et al., 1997). Injecting mice with the antibody blocking c-kit function (ACK-2), the authors concluded that KL/c-kit interaction is important for follicle activation in the first 5 days post-birth (Yoshida et al., 1997). Interestingly, leukemia inhibitory factor [LIF; an interleukin 6 class cytokine that affects the growth and development of cells (Taupin et al., 1998)], is also produced by the granulosa cells as another factor shown to promote primordial to primary follicle transition, increasing KL mRNA production in cultured granulosa cells (Nilsson and Skinner, 2002). LIF may act to induce primordial activation through induction of KL expression, as proved by culture of ovaries in the absence or presence of LIF or neutralizing antibody to LIF in a procedure similar to that described for KL (Nilsson et al., 2002). Similarly, applying the same methodology, ovary culture in absence or presence of a factor and its function-blocking antibody, the stimulatory effect on primordial to primary transition was demonstrated for the factors: (1) basic fibroblast growth factor [bFGF; from FGF family with several roles in development: cell proliferation, migration and differentiation (Ornitz and Itoh, 2001)] and is produced in the oocyte (Nilsson and Skinner, 2001); (2) Bmp4 (growth factor from the TGF-β family member), produced by theca and stromal cells, which also was found important for follicle survival (Nilsson and Skinner, 2003); (3) keratinocyte growth factor [KGF; a fibroblast growth factor member that stimulates epithelial cell proliferation (Rubin et al., 1989)] is also produced by precursor-theca, theca and stromal cells (Kezele et al., 2005) and (4) platelet-derived growth factor (PDGF; a growth factor), interestingly, appears to be produced by the oocyte (Nilsson et al., 2006). Bmp7 (also a growth factor member of the TGF-β superfamily), compared to vehicle, when injected into the ovarian bursa cavity, was also shown to activate primordial follicles and subsequent transition to primary follicle, (Lee et al., 2001).

The presence of three oocyte-specific genes was also shown to be essential for primordial to primary transition: newborn ovary homeobox-encoding gene (Nobox) (Suzumori et al., 2002; Pangas et al., 2004), *Sohlh1* and *Lhx8* (Pangas et al., 2006). Nobox, as the name indicates, is a homeobox gene consequently involved in regulation of development and is expressed in oocytes of primordial, primary and growing follicles (Suzumori et al., 2002). Studies in deficient mice showed an arrest in follicle growth at primordial stage and a total loss of germ cells by day 14 after birth (Rajkovic et al., 2004). Nobox appears to regulate other important oocyte-specific genes such as Oct4 and growth differentiation factor-9 (GDF9; from TGFβ superfamily) (Rajkovic et al., 2004). Both Sohlh1 and Lhx8 are transcriptional factors, which are expressed in germ cell clusters and oocytes of primordial follicles (Pangas et al., 2006). Analysis of female mice lacking the Sohlh1 gene revealed that primary follicles never form and complete depletion of germ cells occurs around 3 weeks post-birth (Pangas et al., 2006). A very similar pattern was encountered for Lhx8-deficient females and because the microarray analysis of Sohlh1$^{-/-}$ ovaries showed a dramatic downregulation of Lhx8, this may be indicative that Sohlh1 is a major regulator of genes involved in folliculogenesis (Pangas et al., 2006). Thus, these three genes are crucial for early oogenesis and folliculogenesis, particularly in primordial follicle activation. Fig. 2 summarizes the factors involved in primordial follicle activation.

Even though, FSH and/or LH influences some of these factors later in folliculogenesis, the transition from primordial to primary follicle is independent of gonadotropins (Buccione et al., 1990; Fortune, 2003). The previously described inhibitors and promoters of primordial follicle activation, produced by either oocyte, granulosa or theca cell precursors, reinforce the importance of cell-to-cell communication and illustrate that primordial follicle activation is a highly coordinated process (Albertini and Barrett, 2003; Skinner, 2005; Hutt et al., 2006b). Oocyte and surrounding granulosa cells are coupled to each other throughout folliculogenesis by gap junctions, which are the most important junctions within the ovary (Anderson and Albertini, 1976; Buccione et al., 1990; Albertini and Barrett, 2003). Gap junctions are intracellular membrane channels that allow sharing of small molecules between adjacent cells (Anderson and Albertini, 1976; Kidder and Mhawi, 2002). These are present in the mouse ovary as early as 17 days post-coitum (Mitchell and Burghardt, 1986). The gap junction consists of groups of 6 protein subunits—connexins—joined together to form a channel, the connexon which is the functional unit of the gap junction; the end of the connexon from one cell docks with the end of the connexon from the adjacent cell to form the gap junction channel (Bruzzone et al.,

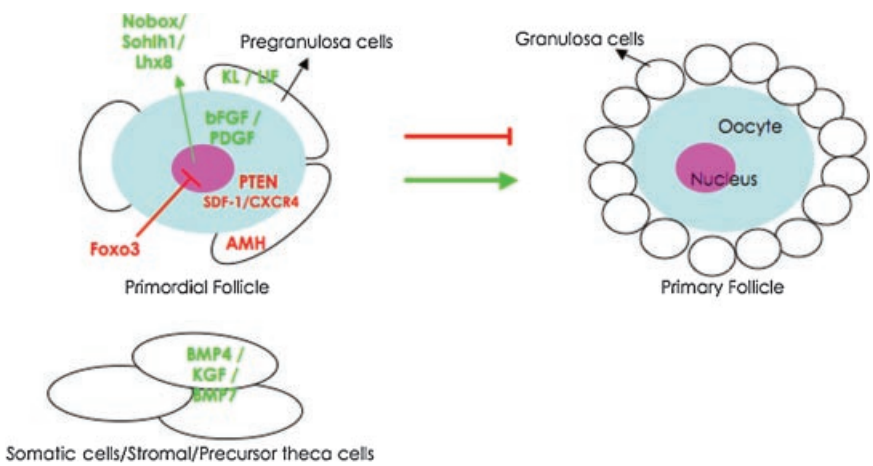

Fig. 2. Schematic representation summarizing the inhibitory and stimulatory factors involved in the primordial to primary follicle transition.

1996). In the mouse ovary, several gap junction proteins have been identified, including connexins (Cxs) 37 and 43, which were demonstrated to be fundamental in folliculogenesis (Kidder and Mhawi, 2002). Connexin 37 is exclusive to the interface between oocyte and granulosa cell. When Cxs 37 is absent, follicle growth arrests at the pre-antral stage (Type 4-see box 3) and although there is some growth of the oocyte it cannot initiate meiotic maturation (Simon et al., 1997; Carabatsos et al., 2000b). Connexin 43 is predominantly expressed in granulosa cells. In mice, targeted mutation of cx43 is lethal. Moreover, Cx43 is involved early in development since a reduced number of germ cells arrive to the genital ridges after Cx43 deletion (Juneja et al., 1999; Granot et al., 2002). When neonatal ovaries were cultured and/or grafted into the kidney capsule of adult females, folliculogenesis was arrested at the primary stage (Juneja et al., 1999; Ackert et al., 2001). Together these results underscore the importance of communication between granulosa cells and the oocyte. $Cx37^{-/-}$ mice support the transition from primordial to primary follicle, however Cx37 must be expressed for subsequent oocyte maturation and continued folliculogenesis (Liu et al., 2006). On the other hand, Cx43 has an earlier role and is required for connections between granulosa cells in order for these to proliferate, form additional granulosa cell layers and support continued follicle growth (Granot and Dekel, 2002; Gittens and Kidder, 2005; Teilmann, 2005; Simon et al., 2006).

Like females lacking Cx43, females of GDF9 null mice exhibited a block in folliculogenesis at the primary follicle stage (Dong et al., 1996). GDF9 is a member of the TGFβ super family expressed only in the ovary (McPherron and Lee, 1993) and found to be exclusively expressed in oocytes beginning in primary follicles and persisting to later developmental stages (McGrath et al., 1995). Interestingly, in hamster GDF9 seems to be essential for primordial follicle formation and granulosa cell differentiation (Wang and Roy, 2006). It was shown that GDF9 stimulates granulosa cell proliferation (Vitt et al., 2000), which might explain the arrest in folliculogenesis of $GDF\text{-}9^{-/-}$ female mice. Aberrant oocyte–granulosa cell interaction was found in these animals, indicating the involvement of the oocyte in somatic cell proliferation, and reinforcing the importance of bidirectional communication between oocyte and granulosa cells and GDF9 paracrine actions in the ovary (Carabatsos et al., 1998; Elvin et al., 1999a,b). Moreover, it has been demonstrated that the impairment in folliculogenesis in $Cx43^{-/-}$ ovaries is in part because the granulosa cells cannot respond adequately to oocyte-derived GDF9 signals (Gittens et al., 2005).

Bidirectional communication is of vital importance to oogenesis and folliculogenesis, as has been shown using GDF9- and Cx43-deficient mice and reviewed by Eppig (2001) and Matzuk et al., (2002). The fundamental structures that provide this connection are the transzonal projections (TZPs), which are extensions from granulosa cells to the oocyte surface that establish and maintain the physical contact between these two cell types (Anderson and Albertini, 1976; Albertini and Rider, 1994; Motta et al., 1994; Albertini and Barrett, 2003). At the end of the TZP, where granulosa cells connect to the oocyte surface, are gap junctions (Anderson and Albertini, 1976; Motta et al., 1994). Interestingly, gap junctions at the end of TZPs are heterotypic composites between Cx43 and Cx37 (Kidder and Mhawi, 2002; Albertini and Barrett, 2003; Teilmann, 2005). Disruption of these gap junctions was found to be detrimental to folliculogenesis, as seen in both Cx37 and GDF9 null mouse models although the relationship between these oocyte-specific genes has not been fully evaluated (Carabatsos et al., 1998, 2000a). Cytoskeletal components of TZPs include both actin-filaments (Act-TZP) and microtubules (MT-TZP), both of which mediate shape and motility of the TZP (Albertini and Rider, 1994; Albertini et al., 2001; Navarro-Costa et al., 2005). Paracrine and hormonal regulation has been suggested as roles of TZPs for the vectorial secretion and/or uptake of signaling molecules at the oocyte–granulosa interface (Albertini et al., 2001; Combelles et al., 2004). These connections are already present in primordial follicles and continue throughout folliculogenesis (Motta et al., 1994; Albertini and Barrett, 2003; Teilmann, 2005). The persistence of a coordinated communication network between oocyte and the surrounding somatic cells ensures ovulation of a healthy oocyte that is ready to be fertilized, the ultimate goal of folliculogenesis and oogenesis (Albertini et al., 2001; Eppig, 2001).

Oocyte hypertrophy: From primary to multilayered follicles

The transition from primordial to primary follicle is prolonged to accommodate the growth phase of oogenesis. Hypertrophy of the oocyte is commensurate with a slow rate of granulosa cell proliferation when the follicle forms a second layer around the oocyte in secondary follicles (Fig. 1D). This protracted proliferative phase increases granulosa cells to six or seven layers in the pre-antral stage (Gougeon, 1996; Fortune, 2003). In the mouse, the appearance of a second layer of granulosa cells is accompanied by zona pellucida formation (Braw-Tal, 2002).

Numerous studies have been done in pre-antral follicles identifying factors responsible for follicle and oocyte growth, but the notion that the oocyte is the driving force for this event has been gaining acceptance (Eppig, 2001; Fair, 2003). In addition to the recognition of GDF9 as an oocyte-specific factor, essential for follicular progression further than primary stage (Dong et al., 1996), another oocyte-specific factor was discovered independently by two laboratories. It is either called bone morphogenetic protein-15 (BMP-15), due to its similarities to the BMP-family (Dube et al., 1998), or GDF9b because of its close homology to GDF9 (Laitinen et al., 1998) (herein referred to as BMP-15). BMP-15 is located on the X-chromosome and has an expression pattern very similar to GDF9 being predominantly in oocytes from primary follicles through ovulation in mouse (Dube et al., 1998) and rat (Otsuka et al., 2000). However, it appears that in some species GDF9 mRNA is present in primordial follicles, particularly in sheep (Galloway et al., 2000), cattle (Bodensteiner et al., 1999), and human (Aaltonen et al., 1999), implying that GDF9 synthesis precedes that of BMP-15. This difference in species-specific expression patterns is interesting and may reflect local signaling requirements in monovular versus litter bearing (multiovular) animals. For example, studies with BMP-15 mutant mice revealed that null females are subfertile, with decreased ovulation and fertilization rates (Yan et al., 2001), but in sheep null BMP-15 females mimic the GDF9 null mouse phenotype in demonstrating follicular arrest at the primary stage (Galloway et al., 2000). At least in mice, this indicates that BMP-15 is more important in later stages of folliculogenesis, whereas GDF9 is needed earlier (Yan et al., 2001). Both factors were proven essential in sheep (Juengel et al., 2002), and their cooperative effect confirmed when recombinant ovine GDF9 and/or BMP-15 were shown to regulate the proliferation of granulosa cells in rat and ruminants (McNatty et al., 2005a,b). Furthermore, it was recently suggested that this cooperation is done through BMP-receptor II (Edwards et al., 2007). Interestingly, GDF9 and BMP-15 interact with KL (produced in the granulosa cells) controlling granulosa cell proliferation (Joyce et al., 2000; Otsuka and Shimasaki, 2002; Thomas and Vanderhyden, 2006). These growth factors have antagonistic roles: GDF9 inhibits KL expression in granulosa cells (Joyce et al., 2000; Wu et al., 2004), while BMP-15 acts as an activator of KL expression in granulosa cells, which in turn inhibits BMP-15 in a negative feedback loop (Otsuka and Shimasaki, 2002; Hutt et al., 2006a). At the same time KL from the

granulosa cell seems to be the theca cell "organizer", inducing interstitial cell recruitment to form the theca layer (Parrott and Skinner, 2000). Evidence for GDF9 inhibition of KL comes from GDF9 null mice that demonstrate up-regulation of both KL and inhibin (Elvin et al., 1999a). Surprisingly, follicles of mice with a double knock-out (KO) for GDF9 and Inhibin-α developed to multilayered stages before ovarian tumors appeared. This indicates that granulosa cells proliferate without both factors, and that the up-regulation of inhibin in GDF9 null alone is responsible for preventing granulosa cell proliferation since its absence is sufficient to promote proliferation (Wu et al., 2004). Several models have been proposed to explain the tight coordination between oocyte growth and granulosa cell proliferation, a time during oogenesis when many essential features of developmental competence are acquired.

One such model invokes coordinate KL and activin up-regulation that drives functional progression during folliculogenesis (Wu et al., 2004). Like activin, inhibin is a member of the TGF-β family and both were initially identified from follicular fluid through their ability to regulate FSH secretion in vitro; inhibin inhibits and activin stimulates FSH production (Ying, 1988; Knight and Glister, 2001; Knight and Glister, 2003; Lin et al., 2003). Follistatin, another component isolated from follicular fluid, is a single-chain glycoprotein with homology to α- and β-subunits of the inhibin/activin family that inhibits FSH production (Ueno, 1987). The regulatory action of follistatin over FSH comes from its capacity to bio-neutralize activin due to high affinity binding to this factor (Nakamura et al., 1990; de Kretser et al., 2002). These three regulators are expressed in granulosa and theca cells of antral follicles and in luteinized granulosa cells, having a weak expression in smaller follicles (Roberts et al., 1993). In fact, it was suggested that activin from bigger follicles stops smaller follicles from growing (Mizunuma et al., 1999). Evidence that these are important factors in the gonadotropin-dependent phase of folliculogenesis includes the facts that there is a 50% reduction in inhibin, and significantly reduced expression of activin in FSHβ-deficient mice (Burns et al., 2001). These mice are infertile arresting follicular development prior to *antrum* formation (Kumar et al., 1997). FSH action is mediated by receptors in the granulosa cell membrane, and in accordance with FSH action of inducing antral follicle formation, female mice with no FSH-receptor (FSHr) gene also exhibited a block at pre-antral follicle stage and were infertile (Dierich et al., 1998; Abel et al., 2000). In these animals both inhibin and activin, were undetectable in serum and in gonads (Abel et al., 2000). Insulin-like growth factor-1 (IGF-1) has been described as having a similar function as activin and regulates FSHr, since it was shown to increase FSHr in granulosa cells (Zhou et al., 1997). Previously, it was demonstrated that IGF-1 is selectively expressed in granulosa cells of healthy small pre-antral and antral follicles, and in the mural and cumulus cells of pre-ovulatory follicles (Oliver et al., 1989). The absence of the IGF-1 gene has a similar phenotype in the ovary as the loss of FSH; folliculogenesis does not proceed further than large pre-antral stage, and FSHr expression is significantly reduced (Zhou et al., 1997). Another suggested regulator of FSH in small pre-antral follicles is AMH, previously shown to inhibit primordial follicle activation (Durlinger et al., 1999). AMH action towards FSH was investigated due to the increased number of pre-antral and small antral follicles found in the ovary of adult AMH null mice despite the low level of FSH in serum (Durlinger et al., 2001). Results suggested that AMH has an inhibitory effect on FSH, balancing pre-antral follicle growth with FSH (Durlinger et al., 2001). These elaborate feedback and feedforward mechanisms likely evolved to support the growth phase of oogenesis through the symbiotic relationship of oocytes and granulosa cells.

Interestingly, FSH appears to serve as a master regulator of oocyte–granulosa cell communication as it has been shown to be capable of reversibly inducing TZP retraction (Albertini et al., 2001; Combelles et al., 2004). As emphasized previously, TZPs are important regulators of oocyte–granulosa cell communication, and the fact that the high number of TZPs observed in oocytes from FSH-deficient females was significantly reduced with the addition of exogenous FSH is indicative of this coordination (Combelles et al., 2004). This coordination also implicates FSH action in maintenance of oocyte growth since oocytes depend on the metabolism of the granulosa cells for most of its macromolecular synthesis (Plancha et al., 2005).

The fact that granulosa cells in pre-antral follicles synthesize inhibin/activin, follistatin, and AMH and are capable of responding to FSH at a critical juncture in oogenesis further supports the importance of mutual signaling (McNatty et al., 2007). See Fig. 3 for a schematic simplification of the factors involved in multilayered follicle formation.

Antrum formation: Antral follicles and ovulation

The continuous proliferation of granulosa cells leads to the production and accumulation of fluid forming small cavities within the layers—early antral follicles (Fig. 1E); eventually the cavities coalesce to become one big fluid filled cavity, the antrum—antral follicles (Fig. 1F) (Peters and McNatty, 1980). In antral stage depending on their location granulosa cells can be either *cumulus* cells, if closely surrounding the oocyte, or mural cells, if adjacent to the follicular wall (Peters and McNatty, 1980).

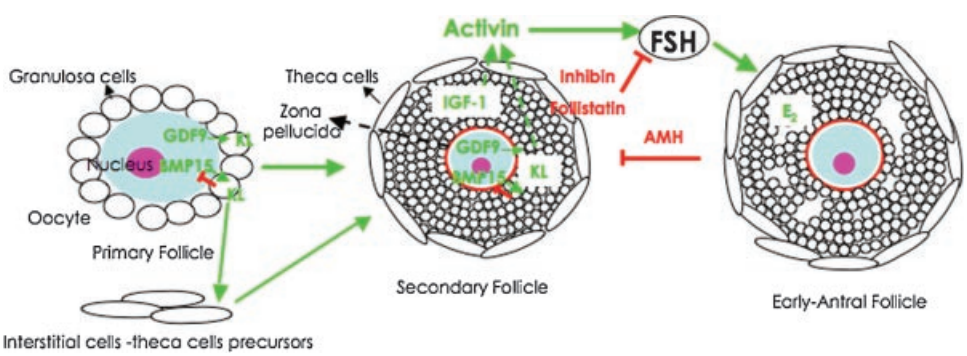

Fig. 3. Schematic drawing of factors involved in multilayered follicle and early fluid-filled cavity formation.

Even though small pre-antral follicles are responsive to FSH this hormone is not necessary for granulosa cell proliferation and follicle growth (McGee et al., 1997). However the second phase of folliculogenesis is hormone dependent involving a complex process essential to the primary goal—ovulation of a fertilizable oocyte. Granulosa cell responsiveness to FSH marks the initiation of the second phase of folliculogenesis and it is dependent on FSHr, which is regulated by IGF-1 (Zhou et al., 1997). In addition to IGF-1, estrogen acts in a supporting role to FSH in granulosa cell proliferation (Richards, 2001; Britt and Findlay, 2002). Estrogen is an endocrine steroid hormone produced by the granulosa cells, which also enhances FSH and induces granulosa cell differentiation (Britt and Findlay, 2002; Drummond, 2006). Estrogen is the product of the steroidogenic pathway (steroid biosynthesis through progressive carbon loss from cholesterol) to estradiol-17β (E_2); higher amounts of E_2 are the major feature of the dominant follicle(s) (Hinshelwood et al., 1994; Baker and Spears, 1999; Drummond, 2006). Like every molecule, estrogens act through their receptors—estrogen receptors (ER) and the ovary has two subtypes ERα and ERβ. Despite the latter's greater abundance in the granulosa cells, the KO female mouse for ERα is infertile, with an arrest in folliculogenesis at antral development. On the contrary, ERβ null mice are subfertile (Couse et al., 1997; Krege et al., 1998; Couse and Korach, 1999) indicating that estrogen binds more ERα for proliferation (granulosa cells), and ERβ is probably used more in differentiation to stop proliferation and initiate necessary changes for ovulation (Britt and Findlay, 2002). The last step in E_2 production is catalyzed by aromatase, a member of the family of genes known as cytochrome P450 (Hinshelwood et al., 1994). When aromatase is not present, ovaries exhibit a phenotype similar to the ERα KO, follicular arrest at antral stage, indicative of the importance of both in folliculogenesis (Fisher et al., 1998). Aromatase is also regulated by FSH as demonstrated by the more than 6-fold decrease of P450 aromatase mRNA in the ovary of FSH-β KO females (Burns et al., 2001). Binding of FSH to its receptor in granulosa cells activates the cyclic adenosine monophosphate (cAMP)/protein kinase A (PKA) pathway, as well as other kinases such as serum and glucocorticoid-induced kinase (Skg) and PKB (protein kinase B)/Akt, downstream targets of the IGF-1/PI3K/phosphatidylinositol-dependent kinase 1 (PDK1) pathway (Gonzalez-Robayna et al., 2000; Richards, 2001). In agreement is the fact that in the FSHβ KO ovary Skg is significantly reduced (Burns et al., 2001). Interestingly, Skg has been implicated in proliferation of granulosa cells as well as their terminal differentiation into luteal cells (Alliston et al., 2000). Evidence for this FSH-cAMP/PKA pathway induced proliferation was demonstrated by studies on cyclin D2 null females, which have impaired folliculogenesis and no ovulation occurs due to abnormalities in granulosa cell growth (Sicinski et al., 1996). As a downstream effector in this pathway, IGF-1-induces granulosa cell proliferation and stimulates LH receptors (LHr) in granulosa and theca cells (Magoffin and Weitsman, 1994) underscoring the importance of IGF-1 and E_2 interaction with FSH in folliculogenesis, initiating the period when granulosa and theca cells are responsive to LH (Richards, 2001; Richards et al., 2002). The key role of LH in folliculogenesis is evident in the study of mice with LH β-subunit disruption where no ovulation and no *corpora lutea* (CL) occurred (Ma et al., 2004). In addition, females lacking LHr develop early antral follicles, but no pre-ovulatory follicles or CL (Lei et al., 2001; Zhang et al., 2001). Therefore, it is not surprising that LH KO females have low serum levels of E_2 and the steroid progesterone (Ma et al., 2004), which is produced by the theca and luteal cells under LH influence (Drummond, 2006). In normal mice, E_2 production by granulosa cells in response to FSH and LH induces a massive wave of proliferation among the granulosa cell population resulting in the formation of large pre-ovulatory follicles (Hirshfield, 1991; Robker and Richards, 1998). Once granulosa cells have acquired LH responsiveness (via LHr), theca cells produce increasing amounts of androgens (cholesterol sub-products prior to estrogens), the LH surge happens and the pre-ovulatory follicle activates a cascade of signaling proteins, which induce major transformation of follicular cells. They stop proliferation (cell cycle) and terminal differentiation (luteinization) begins, oocytes resume meiosis and ovulation occurs (Richards et al., 2002; Mehlmann, 2005).

Throughout follicle growth, the oocyte is arrested in meiotic prophase-I. Before ovulation, meiosis resumes in response to LH (Mehlmann, 2005). However only fully grown oocytes are able to resume meiosis, therefore follicular growth is accompanied by oocyte growth. As the follicle grows and matures, the oocyte volume increases greatly due to massive RNA storage and complex organelle reorganization (Wassarman and Albertini, 1994). Additionally, specific oocyte structures such as the zona pellucida (deposition of the glycoproteins ZP1, ZP2, and ZP3) and cortical granules are formed (Wickramasinghe and Albertini, 1993; Wassarman and Albertini, 1994). The zona pellucida is important not only in fertilization as shown by KO analysis of ZP1 that had reduced litters (Rankin et al., 1999), but also in granulosa–oocyte communication as indicated by genetic disruption of ZP2 and ZP3, which results in mice that ovulate but no zona is present and dramatic alterations are seen at the interface of oocyte and granulosa cell (Rankin et al., 1996, 2001). Cortical granules, on the other hand are small organelles that localize near the oolema of mature oocytes, expelling their contents into the perivitelline space following fertilization and alter ZP properties to block polyspermy (Wassarman and Albertini, 1994).

The oocyte acquires meiotic resumption capacity when follicles become antral (Mehlmann, 2005). This acquisition involves structural rearrangements in the oocyte cytoplasm and nucleus, including cytoskeleton organization. Cytoplasmically, in the meiotic competent oocyte short microtubules (MTs) are enucleated from phosphorylated centrosomes while the incompetent oocyte exhibits long arrays of MTs and non-phosphorylated centrosomes (Wickramasinghe and Albertini, 1992). In general oocyte cytoplasmic maturation is accompanied by loss of MTs and acquisition of multiple microtubule organizing centers (MTOCs) (Mattson and Albertini, 1990). Regarding nuclear rearrangements, incompentent oocytes are characterized by a non-surrounded nucleolus (NSN) chromatin configuration, whereas competent oocytes due to progressive chromatin condensation display a surrounded nucleolus (SN) (Mattson and Albertini, 1990; Albertini et al., 2003). However, in both competent and incompetent oocytes the nucleus is named germinal vesicle (GV). The first of two steps in meiotic acquisition is GV breakdown (GVBD), nuclear envelope breakdown with consequent progression to metaphase I (MI, reduction division), secondly the oocyte gains the capacity to move from MI into MII (metaphase II) (Wassarman and Albertini, 1994). In the mouse, briefly before GVBD MTOCs are activated in the vicinity of the chromosomes and MTs are stabilized and become progressively organized into a bipolar spindle around the chromosomes (Brunet and Maro, 2005).

How Oogenesis Presupposes Embryogenesis

The maternal legacy in organisms with distinct reproductive strategies

Central to an understanding of oogenesis in an organismal context is recognition of the physiological mechanisms that establish the timing of this process. Nutritional status and metabolic stability are two important factors that organisms use to initiate and maintain the process of oogenesis. Thus it is not

uncommon that animals encountering environmental or seasonal restrictions in diet opt to stop or delay oogenesis until appropriate nutritional requirements can be met to support what is often a metabolically taxing process. This basic tenet is the core of oogenesis that defines the role of hypertrophy and storage of organelle precursors that are called upon to support the metabolic demands of embryogenesis. In general, the embryo reaches a stage of self-sufficiency either by having reached a level of feeding competency or by establishing a maternal source of nutrition through the placenta. The second major factor that links oogenesis to embryogenesis is the ability to establish continuity of organelles from one generation to the next. Here the relationship between genes and the environment has taken on increased significance for future studies.

Maternal effectors: From genes to environments

Maternal effectors play critical roles in oogenesis. While this has been appreciated for many years from studies on maternal effector genes in flies and worms, the significance of this to mammalian oogenesis has only recently been demonstrated. As shown in Table 1, there are now many gene deletion models in the mouse that illustrate both classical examples of maternal effector genes (MATER, ZAR1, Dmnt1O) and genes active in either the oocyte or surrounding somatic cells that are required for progression of oogenesis or folliculogenesis. The emerging trend from a compilation of phenotypes implies a form of oocentric regulation at the onset of oocyte growth that is then subject to mutual germ line somatic cell feedback control that invokes both local paracrine elements as well as systemic interactions between the ovary and various somatic tissues (Hutt and Albertini, 2007). Thus, maternal effectors for oogenesis are both genetic and absolute in terms of specific gene products that are required to initiate and sustain embryogenesis. Given the dependency on somatic control in mammals like the mouse, there is every reason to believe that epigenetic factors profoundly

TABLE 1. Mouse mutation and phenotypes that affect oogenesis (early and late)

Gene mutated	Mouse knockout phenotype	Reference
Primordial germ cells—PGCs deficiencies		
B-cell lymphoma/leukaemia 2 (*Bcl2*)	Reduced survival of PGCs	Ratts et al. (1995)
Bcl2-associated X (*Bax*)	Increased follicular endowment	Greenfeld et al. (2007)
Bone morphogenetic protein 4 (*Bmp4*)	No PGCs	Lawson et al. (1999)
Bone morphogenetic protein 8 (*Bmp8*)	Greatly reduced or no PGCs	Ying et al. (2000)
Caspase 4 (*Casp 4*; also known as *Casp11*)	Reduced number of oocytes at birth	Morita et al. (2001)
Connexin 43 (*Gja1*)	Reduced PGCs	Juneja et al. (1999)
Dominant white spotting (*W*), encode for and c-kit	Improper PGC migration and germ cell deficiency	Buehr et al. (1993) (W)
Follistatin (*Fst*)	Early germ cell loss, sex-reversal	Yao et al. (2004)
Octamer-4 (*Oct4*)	Massive apoptosis of PGCs	Kehler et al. (2004)
Peptidyl-prolyl isomerase 1 (*Pin1*)	Impaired germ cell proliferation	Atchison et al. (2003)
Smad 5	Greatly reduced or no PGCs	Chang and Matzuk, (2001)
Still (*Sl*), encode for kit ligand (KL)	Improper PGC migration and germ cell deficiency	Zama et al. (2005) (Sl)
Wnt4	Early germ cell loss, sex-reversal	Vainio et al. (1999)
Primordial follicle (POF) assembly deficiencies		
Aryl-hydrocarbon receptor (*AhR*)	Affects germ cell apoptosis—increased POF numbers	Robles et al. (2000)
Dazla	Complete absence of follicles	Ruggiu et al. (1997)
Factor in germline alpha (*Figla*)	No POF assembly, complete germ cell loss by day 2	Soyal et al. (2000)
Pre-antral follicles deficiencies		
Anti-Müllerian hormone (*Amh*)	Enhanced follicle recruitment	Durlinger et al. (1999)
Connexin 43 (*Gja1*)	Arrest in primary follicles	Ackert et al. (2001)
Connexin 37 (*Gaj4*)	Arrest at pre-antral stage of folliculogenesis and meiotically incompetent oocytes	Simon et al. (1997) and Carabatsos et al. (2000a,b)
Estrogen receptor α (*Esr1*—ERKO)	Arrest of follicle growth at early antral formation	Couse et al. (1997) and Dupont et al. (2000)
Follicle-stimulating hormone (*Fshβ*)	Arrested folliculogenesis prior to antral follicle formation	Kumar et al. (1997)
FSH receptor (*FshKO*)	Arrested folliculogenesis prior to antral follicle formation	Abel et al. (2000)
Follitropin receptor (*FORKO*), all FSH-R variants	No antral follicle formation, fewer germ cells	Danilovich et al. (2001 and 2004)
Forkhead box L2 (*Foxl2*)	No secondary follicles, no granulosa cell differentiation	Schmidt et al. (2004) and Uda et al. (2004)
Forkhead box O3 (*Foxo3*)	Rapid oocyte depletion due to POF recruitment	Castrillon et al. (2003)
Growth differentiation factor-9 (*Gdf9*)	Arrest at primary stage folliculogenesis; no theca layer and incompetent oocytes	Dong et al. (1996) and Carabatsos et al. (1998)
Insulin-growth factor-1 (IGF-1)	Folliculogenesis arrest at multilayered follicles, no antrum formation	Zhou et al. (1997)
Lhx8	Early germ cell loss, no primary follicle formation	Pangas et al. (2006)
Newborn ovary homeobox (*Nobox*)	Deficient POF to primary transition, no germ cells by day 14	Rajkovic et al. (2004)
Sohlh1	No primordial to primary transition, early germ cell loss	Pangas et al. (2006)
Smad3	Impaired folliculogenesis, no antral follicles formed	Tomic et al. (2004)
Ovulation and fertilization deficiencies		
A disintegrin and metalloproteinase with thrombospondin-like motives-1 (ADAMTS-1)	High rate of anovulation	Shozu et al. (2005)
Aromatase-CYP19 (ArKO)	No ovulation due to impaired folliculogenesis	Fisher et al. (1998) and Britt et al. (2001)
Cyclooxygenase-2 (COX-2)	Ovulation failure, no fertilization	Lim et al. (1997)
Cyclin D2	Abnormal GC proliferation, oocytes do not ovulate	Sicinski et al. (1996)
Bone morphogenic protein-15 (Bmp15)	Small litters—reduced ovulation and fertilization rates	Yan et al. (2001)
Estrogen receptor β (Esr2-BERKO)	Reduced number of oocytes ovulated, small litters	Krege et al. (1998)
Inhibin α	Reduced number of oocytes ovulated, small litters	Matzuk et al. (1992)
Luteinizing hormone (Lh)	Infertile, no ovulation	Ma et al. (2004)
LH-receptor (Lhr)	Antral follicles, but no ovulation	Lei et al. (2001); Zhang et al. (2001)
Progesterone receptor (PR)	No ovulation	Lydon et al. (1996)
Zona pellucida 1 (ZP1)	Loose organization of zona pellucida—reduced litters	Rankin et al. (1999)
Zona pellucida 2 (*ZP2*)	Thin zona pellucida that disappears in late folliculogenesis and ovulated oocytes—infertile	Rankin et al. (2001)
Zona pellucida 3 (ZP3)	No zona pellucida and no cumulus expansion—infertile	Rankin et al. (1996)

influence oogenesis. Recent studies on environmental toxins have reinforced this concept in a rat model (Hutt et al., 2008).

Modifiers of developmental competence in mammals

Developmental competence in mammalian oocytes refers to the well-established fact that discrete transitions in the physiology of the oocyte define the acquisition of a functional capacity. For example, the primordial to primary oocyte transition initiates an overt hypertrophic condition without cell cycle resumption from meiosis I arrest and therefore is analogous to the G2 stage of the somatic cell cycle (Wickramasinghe and Albertini, 1993). As noted above, this transition is modified by many factors of oocyte and granulosa cell origin (see Table 1). This is followed sequentially by the acquisition of meiotic competence, the competence to initiate appropriate fertilization responses like the cortical reaction and exit from meiosis 2, and the competence to affect early mitotic cell cycles in the embryo. Notably, each of these competencies is acquired at specific stages of folliculogenesis. Thus, modifiers of a very diverse nature are likely to impact the developmental competencies of an oocyte. Current work in this area is focused on maternal modifiers that include age, hormonal or nutritional status of females as well as direct or indirect consequences that result from environmental exposures [e.g. bisphenol A (BPA) (Susiarjo et al., 2007)] or following therapeutic interventions for fertility or cancer. The conclusion here is that there are high risks in organisms such as mammals that exhibit prolonged segments of their pre- and post-pubertal lifespan that modifiers of oogenesis will impact the developmental competence of oocytes and embryos without necessarily interfering with the completion of pregnancy. Understanding and defining those segments of female lifespan that are at greatest risk for genetic or epigenetic modification of oogenesis is a major and timely challenge that lies ahead (Bromfield et al., 2008).

Strengths and Limitations of Animal Models

Intrinsic versus extrinsic control

Comparative physiology provides a useful forum for validating and extending the utility of animal models for understanding human disease. The field of oogenesis has benefited enormously from the use of animal models, but parallel advances in humans and mammals other than the mouse call into question the translational significance of this line of study. Specifically, the relative contribution of factors intrinsic or extrinsic to the process of oogenesis that ensure oocyte developmental competence has been largely ignored. What might these factors be? Nutrition, environmental exposure, and reproductive lifespan have already been implicated as extrinsic factors that would directly or indirectly influence ovarian physiology. Oocyte intrinsic gene expression has similarly been identified via gene targeting or RNA knockdown strategies. But the confluence of these two broad categories of regulatory principles has yet to be fully appreciated and may reside in the physiological regulation between the germ line and somatic cells with which the oocyte is in direct contact. It is with this in mind that a comparative analysis may be helpful in defining the utility of animal models.

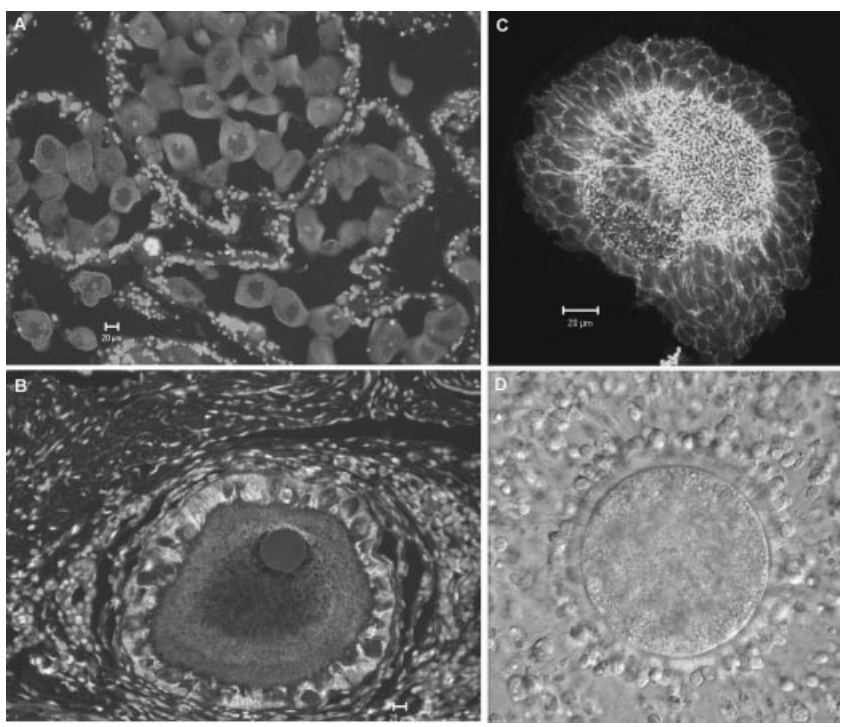

Fig. 4. Micrographs depicting variety of oocyte somatic cell interactions observed in (A) surf clam, (B) ray, (C) canine, and (D) bovine. Note the limited germ cell contact in surf clam (A), where oocyte clusters are released from outpocketings of the coelomic epithelium; in highly vitellogenic species like the ray (B), a single layer of follicle cells labeled with acetylated tubulin antibodies is depicted in which apical projections approximate the oolemma. In mammals (C and D), subsets of granulosa cells are anchored to the zona pellucida that project many thousands of actin-containing transzonal projections (Act-TZPs) making direct physical contact with the oolemma. (C) Confocal projection of canine cumulus oocyte complex before ovulation stained with rhodamine phalloidin to illustrate the density of f-actin projections at the zona surface. (D) DIC image of a bovine cumulus complex after ovulation in which many projections are retained within the zona pellucida that is surrounded by a single layer of granulosa cells known as the corona radiata. Scale bars 20 μm.

Flies, worms and mice at the front edge

Fig. 4 serves to illustrate the extreme variability in the basic design pattern at the interface of oocytes with follicle cells. Highly seasonal spawners like marine invertebrates tend to modify the coelomic epithelium into extensive folds of germinative potential accounting for much of an animals' metabolic energy for egg production. A role for follicle cells emerges in organisms that invest in vitellogenesis more selectively by producing relatively fewer developmentally competent oocytes and establishing defined physical inputs from the surrounding somatic cells (Fig. 4B). The amplification of cell contact between follicle cells and oocytes is a hallmark of virtually all eutherian mammals (Fig. 4C and D). Enhancement of the communication opportunities between the soma and germ line is not necessarily a carry over from the need to provide yolk precursors to the growing oocyte and instead seems to be a reflection of the growing need to monitor and promote a sensing system that titrates both somatic and germ line health. If such a concept holds up, it will be important to consider the varying demands for oogenesis in model systems that use stem cells to propagate new oocytes (flies, worms), or that deploy relatively short pre-pubertal stages to establish a small fixed supply of oocytes that would last the entire reproductive lifespan (mouse). These model organisms remain mainstays in the field of oogenesis research and will continue to contribute invaluable genomic and epigenomic information with which to further explore the problem of oogenesis. They may not, however, be the most opportune for understanding human disease.

Future model systems

From the above discussion, it is clear that if alternative model systems were available for purposes of clinical translation, a discrete boundary could be drawn between the investigative utility of model systems and the pressing needs emerging from the fields of human stem cell and ART research. In this regard, the shortcomings of material shortage, ethical issues, and experimental tractability have often been raised as impairments to research on the biology of oogenesis in humans. This landscape is changing. Opportunities to store ovary, oocytes, and embryos have increased as cryopreservation technology has advanced this field from the mouse to human materials. Using unfertilized parthenogenetically activated oocytes bypasses some ethical concerns but may ironically add a new twist to this debate in validating the immaculate conception. And finally, bioinformatics resources and approaches are supplying the necessary foundation for asking direct questions about human oocytes that will aid in understanding the defects and deficiencies that underscore female reproductive health.

Conclusions

This review will hopefully have served two purposes: (1) to highlight the complexities inherent in oogenesis that need to be solved to understand quality egg production and (2) to draw timely comparisons between various animal models with respect to their utility in addressing the problem of maternal inheritance and totipotency. Oocytes in mammals are rare and highly specialized cells whose livelihood holds the fate of future generations. They are also very culpable cells subject to the whims and fancies of their somatic surrounds and as such are targeted for modification or destruction by lifestyle, misguided immune regulation, environmental toxins, hormones, and medications that are being used increasingly in immune suppression or chemotherapy. Model systems will continue to provide a wealth of information bearing on fundamental aspects of the process of oogenesis but extrapolations to mechanisms that bear on human or animal health will require a species-specific approach. Thus, protecting the female germ line will necessitate that experimentalists, clinicians, public health specialists, and ethicists synergize their efforts in devising acceptable standards and approaches for research on the biology of human oocytes.

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Multiple mechanisms of germ cell loss in the perinatal mouse ovary

Patricia Rodrigues[1,2], Darlene Limback[1], Lynda K McGinnis[1], Carlos E Plancha[2] and David F Albertini[1,3]

[1]*Department of Molecular and Integrative Physiology, Center for Reproductive Sciences, 3088 Kansas Life Science Innovations Center, University of Kansas Medical Center, KLSIC Room 3016, 3901 Rainbow Boulevard, Kansas City, Kansas 66160, USA,* [2]*Unidade de Biologia da Reprodução, Instituto de Medicina Molecular and Instituto de Histologia e Biologia do Desenvolvimento, Faculdade de Medicina da Universidade de Lisboa, Avenue Professor Egas Moniz, 1649-028 Lisbon, Portugal and* [3]*Marine Biological Laboratories, Woods Hole, Massachusetts 02543, USA*

Correspondence should be addressed to D F Albertini; Email: dalbertini@kumc.edu

Abstract

In the perinatal ovary of most mammals, external and internal factors establish a primordial follicle reserve that specifies the duration of the reproductive lifespan of a given species. We analyzed the mechanism of follicle loss and survival in C57BI/6 mice using static and dynamic assays of apoptosis, autophagy, and ovarian morphogenesis. We confirm an initial loss soon after birth, when about 44% of the germ cells detectable at the end of the fetal period abruptly disappear. The observations that (1) few germ or somatic cells were apoptotic in newborn ovaries, (2) vitally stained organ cultures exhibit active extrusion of non-apoptotic germ cells and (3) germ-cell lysosome amplification occurs at birth suggested that additional mechanisms are involved in perinatal germ cell loss. Newborn mouse ovaries cultured in the pH sensitive dye lysotracker red exhibit an increased incidence of acidified non-apoptotic germ cells when maintained in the absence but not in the presence of serum, implying a role for autophagy in germ cell attrition. Inhibitors of autophagy, but not apoptosis, reduce germ cell acidification induced by serum starvation in ovary organ cultures and protein mediators of both autophagy and apoptosis are expressed at birth. From these findings we suggest that multiple perinatal mechanisms establish the primordial follicle reserve in mice.

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Introduction

The transition from fetal to neonatal life is a critical phase of normal development in eutherian mammals during which organ systems adapt to post-parturition starvation. Many organ systems in newborns adjust progenitor cell density and function by engaging apoptotic and non-apoptotic mechanisms of programmed cell death (PCD; Edinger & Thompson 2004). There are three types of PCD. Type I, or apoptosis, is characterized morphologically by nuclear condensation and fragmentation, cell shrinkage and membrane blebbing (Tilly 2001, Edinger & Thompson 2004). Type II, or autophagy, is a lysosomal degradation pathway that involves formation of autophagic vacuoles during periods of massive cell elimination (Edinger & Thompson 2004, Qu *et al*. 2007). Type III, or necrosis, is characterized by plasma membrane breakdown causing an inflammatory reaction (Edinger & Thompson 2004). Apoptosis is coupled to autophagy as a means to maintain tissue viability and energy homeostasis in developing tissues during the demanding neonatal phase in mammals (Kuma *et al*. 2004, Yu *et al*. 2004, Lum *et al*. 2005). This coupling occurs either via constitutive pathways, where lysosomes degrade the apoptotic bodies, or through an alternative pathway in which lysosomal proteases such as cathepsins, trigger apoptosis (Bursch 2001, Guicciardi *et al*. 2004). Amongst the organ systems that exhibit profound cell loss at birth is the ovary, where it has long been recognized that massive female germ cell attrition *post partum* precedes the establishment of a fixed follicle reserve that is progressively depleted during the reproductive lifespan (Reynaud & Driancourt 2000, Tilly 2001, Qu *et al*. 2007). Studies favoring a major role for germ cell apoptosis include those using ubiquitously targeted deletion of genes involved in Type I PCD. Accordingly, an increase or decrease in follicle numbers has been reported in postnatal mice bearing systemically targeted gene deletions for anti-apoptotic, or pro- apoptotic genes (Perez *et al*. 1999). Although apoptosis is believed to account for the bulk of germ cell loss in fetal mouse ovaries (Coucouvanis *et al*.

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1993, De Pol *et al*. 1997, Pepling & Spradling 2001), other processes such as germ cell extrusion (Wordinger *et al*. 1990) and autophagy also participate in pre- and postpartum adjustments of germ cell numbers (Wordinger *et al*. 1990, Lobascio *et al*. 2007). Both apoptosis (Edinger & Thompson 2004, Maiuri *et al*. 2007) and autophagy (Maiuri *et al*. 2007), lysosome-mediated PCD processes (Guicciardi *et al*. 2004), have been proposed to serve cooperatively during developmental transitions in other organs, but this prospect has yet to be studied during perinatal ovarian germ cell loss.

The present study addresses several questions related to morphogenesis of the mouse ovary revealing a previously unappreciated level of complexity in PCD that sets and maintains adequate numbers of follicles for reproductive function in mice without invoking replacement strategies. Our findings suggest that multiple mechanisms including autophagy mediate germ cell loss and are used in a coordinated and developmentally regulated fashion.

Results

Characterization of germ cell number during ovarian development

Given the wide range of variability reported for germ cells during ovarian development in mice, we adopted a technical modification for our morphometric analyses. Here, histological sections processed for detection of mouse vasa homologue (MVH), a specific germ cell marker (Fujiwara *et al*. 1994) combined with periodic acid Schiff's (PAS) staining so as to render extracellular matrix, including the zona pellucida, clearly discernible (Fig. 1). Sections of ovaries from C57Bl/6 females ranging in age from embryonic day (E) 15.5 through to postnatal day (P) 150 were morphometrically analyzed for total oocyte, follicle, and/or zona pellucida profiles (Fig. 2 and Supplementary Table 1, which can be viewed online at www.reproduction-online.org/supplemental/). Examples of embryonic, postnatal, and prepubertal ovaries are shown in Fig. 1 to emphasize the striking histological transformation that takes place over these key developmental stages that include resolution of ovigerous cords into solitary follicles (Fig. 1A and B) and expansion of somatic cells including development of the vasculature (Fig. 1C).

In this study, we confirm the decrease in mean germ cell numbers per ovary at birth, with 5783 ± 475 (confidence intervals (CI): 4463–7103) total follicles observed prior to birth (E19.5) and 3252 ± 820 (CI: 976–5529) total follicles after birth (P2; Fig. 2 and Supplementary Table 1). Variations in mean oocyte number were also evident from immediately after birth through to the onset of reproductive cyclicity (data not shown). It should be emphasized that although germ cell number between P2 and P20 remains unchanged, considerable variability is observed between animals as expressed by the wide CI found for the mean numbers of total follicles at ages P2 (CI: 976–5529), 12 (CI: 264–4996) and 20 (CI: 947–5399; Fig. 2). In sharp contrast to the lack of stability in germ cell number observed pre-pubertally, the rate of decline in follicle number from the onset of cyclicity (P42, CI: 1830–3713 total follicles) through adulthood is consistent between time points and animals. This was further confirmed by performing similar analysis on animals in which stages of the estrous cycle were monitored (data not shown). Again, germ cell counts, *corpora lutea* and atretic follicles were similar at the age evaluated (P100, CI: 1419–2579 total follicles). Furthermore, estimates of the total number of zona pellucida remnants as a function of animal age also showed that their appearance coincided with the onset of reproductive cyclicity, from undetectable at P20 to 20.8 ± 3.2 at P42 and 34.9 ± 1.7 at P100 (mean number per ovary for $n = 5$ animals for each time point). These

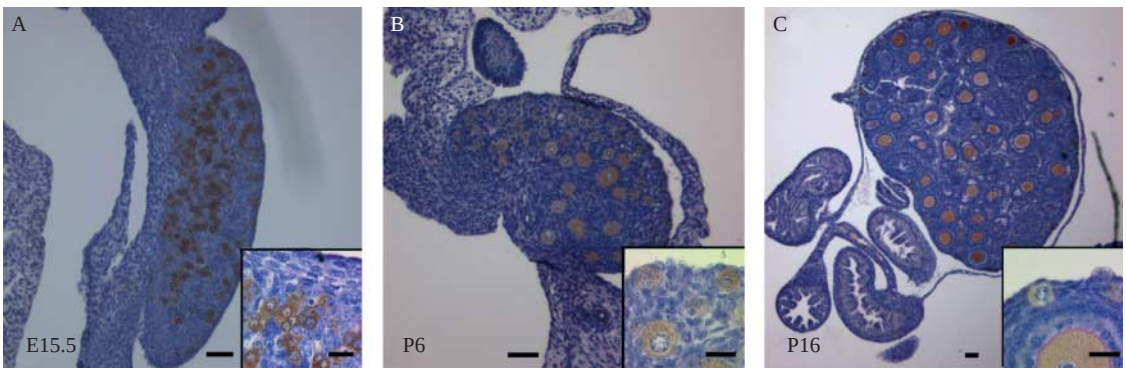

Figure 1 Germ cell disposition during ovarian histogenesis. MVH immunocytochemistry (brown reaction product) defines developmental variance in germ cell density and location. (A) Embryonic day 15.5 ovary, showing germ cell clusters and relative paucity of somatic tissue (inset). (B) Postnatal day 6 ovary, with cortical primordial follicles and medullary primary follicles; note apposition of peritoneal mesothelium to ovarian surface defining bursal cavity. (C) Postnatal day 16 ovary illustrating onset of antrum formation in centrally located follicles and cortically disposed preantral follicles with growing oocytes; inset shows 2 primordial follicles near surface and subtended by a primary follicle. Scale bars = 40 μm for A, B and C, whereas for insets scale bars = 20 μm.

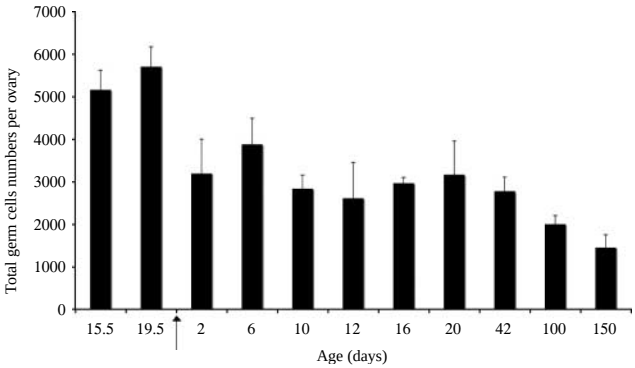

Figure 2 Mean number of total germ cell number per ovary from embryonic day 15 (E15.5) through to adult day 150 (P150). Note marked decrease in mean germ cell number following birth ($P<0.05$), and animal-to-animal variance in oocyte density between days P6 and P20 of age. Onset of cyclicity (P42) coincides with decrease in mean germ cell numbers that steadily declines up to P150. Data are presented as mean ± S.E.M. ($n=5$ animals per age group). Arrow indicates day of birth (day 20 *post coitum*).

results are consistent with little if any net change in follicle number during prepubertal life and therefore prompted further enquiry into the nature of germ cell loss at perinatal developmental transitions.

Germ cell apoptosis during pre- and postnatal ovary remodeling

We first investigated the expression of various apoptosis markers prior to and following birth. Two common markers for early apoptosis, active caspase 3 and cleaved poly (ADP ribose) polymerase (PARP), were evaluated and the more traditional TUNEL assay for terminal stages of apoptosis. E15.5 and E19.5 samples exhibited limited staining for all markers in germ and somatic cells (Fig. 3A–C). This relative paucity of apoptotic germ cells was also evident in postnatal samples (Fig. 3D–F). As shown below, large clusters of germ cells were often seen at the surface of P2 ovaries, but even these clearly discarded oocytes were only rarely found to be positive for apoptotic markers. In contrast, ovaries from adult cycling females exhibited the apoptosis patterns well established for mammalian ovary (Fig. 3G–I), with granulosa cells (GCs), but not oocytes in atretic follicles positive for apoptosis markers. The rarity of apoptotic germ cells prior to and following birth is difficult to reconcile with the 40% reduction seen by us and others and prompted investigation of alternative cell death mechanisms.

Postnatal ovarian histogenesis and germ cell extrusion

We first evaluated germ cell extrusion or shedding, as a mechanism of germ cell loss. Oogonia and primordial follicles were scored based on their position relative to the ovarian epithelium; these were classified as extra-ovarian if in the bursa cavity or intra-epithelial if positioned at the ovarian surface (Fig. 4A–D). As summarized in Fig. 4E, the mean number of extra-ovarian oogonia/oocytes observed in the bursa account for a minor fraction (~2%) of the total germ cells recorded at these time points. Intra-epithelial oogonia/oocytes represent a transient population of about 300 follicles at each stage examined up to P42, after which time they are not detected (Fig. 4E). Because of the difficulty in determining the flux rate of oocyte extrusion in fixed samples, we conducted live imaging studies in ovarian organ cultures. Intact E19.5, P0 (day of birth), and P1 ovaries were cultured for 4 or 18 h. Germ cells associated with the ovarian surface were large and spherical and rarely contained associated somatic cells (Supplementary Figure 1, which can be viewed online at www.reproduction-online.org/supplemental/) whereas those that accumulated on the bottom of the culture dish after 4 h in culture were found in clusters of 4–8 cells (Supplementary Figure 1). While active extrusion was observed at ages examined, attempts to calculate a rate of loss were unsuccessful. An estimated extrusion rate of 150 germ cells per ovary over an 18 h culture interval for P0 ovaries was obtained (Supplementary Figure 1) but in the absence of a way to follow single germ cells, we conclude that this mechanism of loss accounts for only a minor fraction of the loss observed until a more systematic analysis can be performed.

Lysosome compartment amplification and autophagy

Since lysosomes are involved in both autophagy and apoptosis (Guicciardi *et al*. 2004), we first analyzed their expression in somatic and germ cells of perinatal ovaries. Using LAMP1 antibody, which detects a membrane constituent of functional lysosomes, a striking increase in lysosome density was observed from E19.5 to post-natal ovaries (Fig. 5A and B). Figure 5C and D show further that the increase in LAMP1 staining in germ cells coincides with the onset of oocyte growth and follicle assembly. Quantification of LAMP1 by image analysis of stained ovarian sections further confirmed the lysosome amplification post-birth (Fig. 5E). Significant changes in the amplification of lysosomes were observed from E19.5 2.02 ± 0.21 (CI: 1.60–2.43) to P2 15.08 ± 0.95 (CI: 13.19–16.19) by measuring the mean LAMP1 staining per germ cell. The general pattern of increased lysosome expression after birth was also seen with the vital lysosome marker acridine orange (data not shown). Thus, lysosome amplification in all oocytes occurs upon birth and as in other postnatal tissues may represent an adaptive response to postnatal starvation. To directly address this question, an additional experimental strategy was used to ascertain the relative contributions of apoptosis and autophagy in the perinatal mouse ovary.

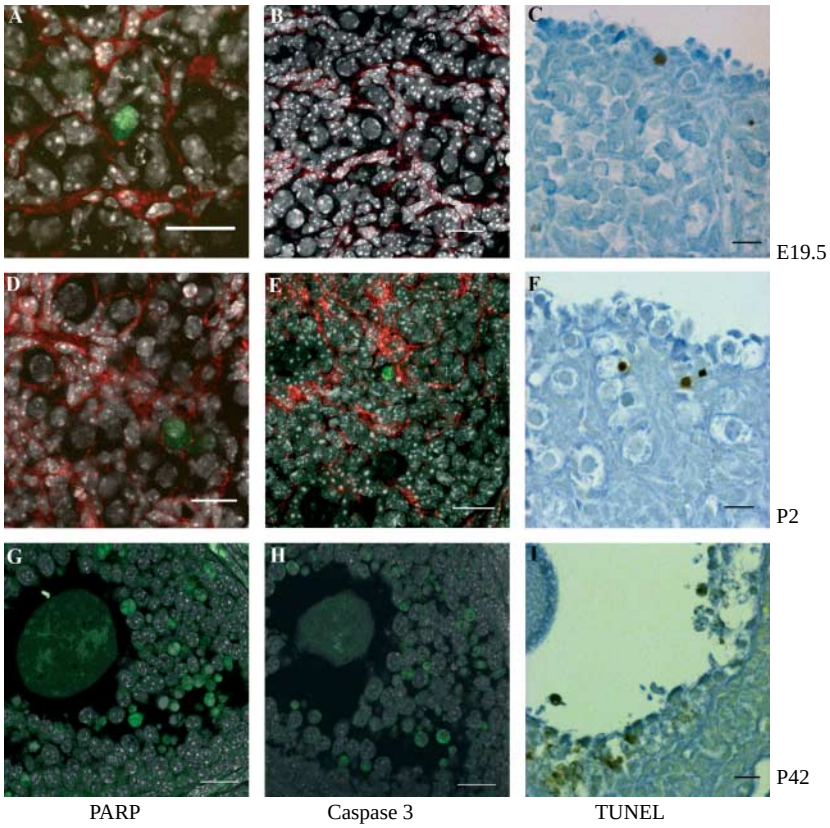

Figure 3 Pre- and postnatal apoptosis patterns. Comparison of apoptosis detection by cleaved-PARP (A, D, and G), active caspase 3 (B, E, and H) or TUNEL (C, F, and I) assays in E19.5 (A, B, and C), P2 *post partum* (D, E, and F), and P42 (G, H, and I) C57Bl/6 ovaries. (A) Confocal optical section of intact E19.5 ovary showing a solitary cleaved-PARP positive (green) germ cell amongst many negative germ cells (f-actin red, nuclei white). (D) Confocal optical section of intact ovary (P2) showing absence of staining in germ (one exception) or somatic cells for cleaved-PARP. Also note rare TUNEL positive cells in comparable ovaries (B, E). (G) (PARP red, GDF9 green), (H) (caspase red) and (I) (TUNEL brown) show that under identical processing conditions, apoptosis is readily demonstrable in granulosa cells of antral follicles in day P42 ovary. Scale bars = 20 μm.

We adapted the methods of Zucker *et al.* (1998) to image intact ovaries that had been labeled with the probe LysoTracker Red (LTR) using high-resolution confocal microscopy to monitor cytoplasmic and organellar acidification in tissues in an unperturbed state. In E19.5 ovaries, LTR reveals smaller apoptotic cells, macrophages identified by multiple lysosomes, and larger germ cells evidenced by acidified cytoplasm (Fig. 6A and B). Acidified LTR labeled germ cells were common at P2, but rare at later stages of ovarian

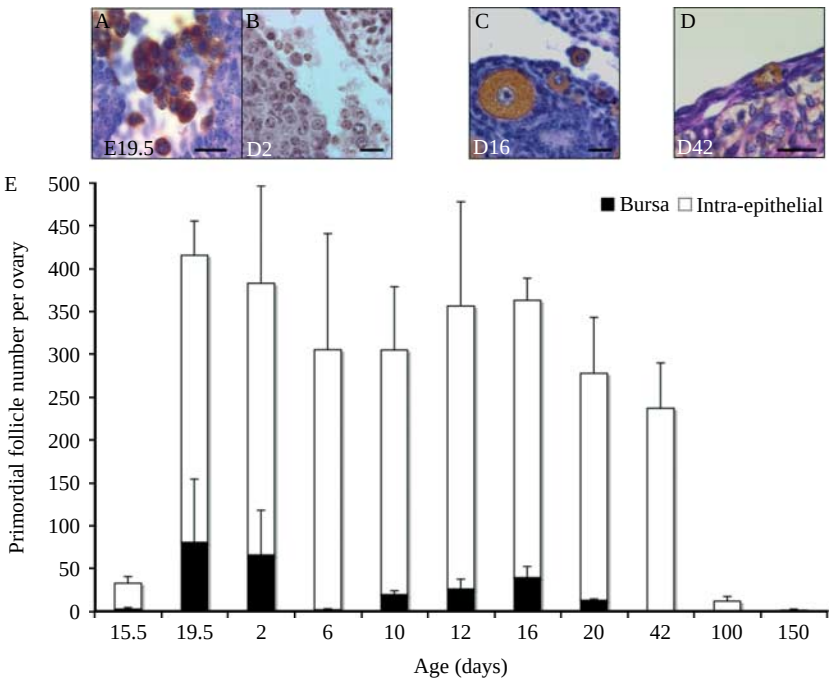

Figure 4 Developmental pattern of germ cell extrusion in mouse ovary. Germ cells were classified as extra-ovarian when within the bursal cavity (A–C) or intraepithelial when associated with or just beneath ovarian surface (D). (A–D) Bright field images of sections stained with MVH (brown), hematoxylin (blue) and PAS (pink). Bars = 20 μm. (E) Comparison of extra-ovarian (filled bars) or intraepithelial (open bars) germ cells over developmental age range studied (E15.5–P150). Mean number primordial follicles ± S.E.M. per time point are plotted ($n = 5$ ovaries/age).

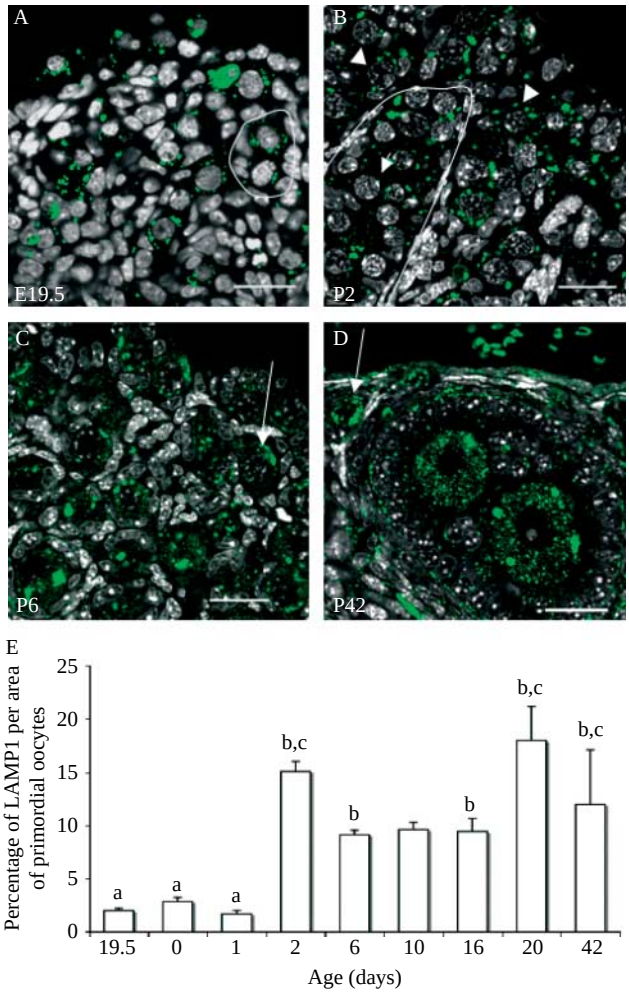

Figure 5 LAMP1 expression in neonatal mouse ovaries. Prenatal ovaries (A and B) exhibit LAMP1 stained lysosomes (green) predominantly in germ cells (somatic and germ cell nuclei are white). After birth, large lysosomes are evident in oocytes that form primordial follicles (C–D, arrows) whereas smaller lysosomes within oocytes that are not fully enclosed by follicle cells (B, arrowheads). (E) Shows quantitation of LAMP1 staining in oocytes between E19.5 and P42; note the abrupt increase at P2 and stabilization of lysosome density from P16 on. Columns with different letters are significantly different from each other ($P<0.05$). Scale bars = 20 μm.

development (Fig. 6C and D). We next tested the effect of serum starvation on the loss of germ cells in an ovarian organ culture system.

To accomplish this, ovaries from P0 animals were cultured and exposed to LTR in the presence or absence of serum for 24 h. Complete Z stack projections were thresholded to demarcate acidified germ cells (Fig. 7A–C and Supplementary Figure 2, which can be viewed online at www.reproduction-online.org/supplemental/), which were counted and expressed as mean density volume per ovary. Direct comparison between P0 (56.93 ± 8.86; CI: 18.80–95.08) and P1 (mean of 20.99) for ovaries showed a 60% decrease at these time points with freshly isolated tissues. Comparison of

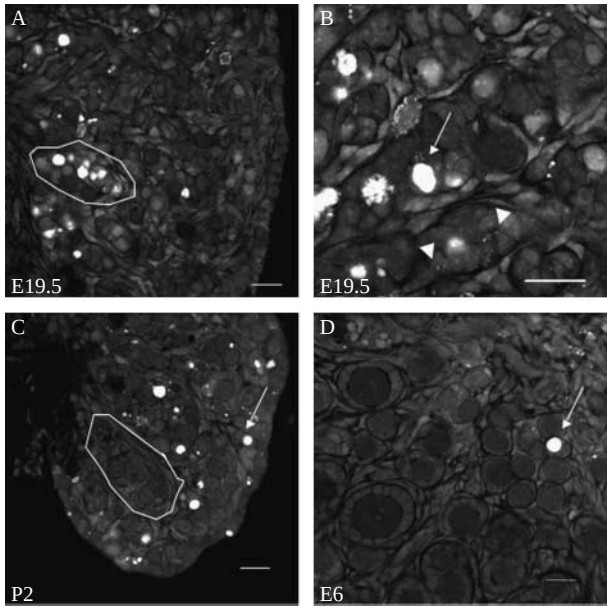

Figure 6 Analysis of LTR staining in intact mouse ovaries. E19.5 ovaries (A and B) just prior to birth containing both cytoplasmic acidification (arrows) and LTR foci (arrowheads) are seen within germ cell cluster (some circled for easy visualization). From P2 (C) to P6 (D), seem to have fewer cells with cytoplasmic acidification (arrows). Scale bars = 20 μm.

serum treated (14.40 ± 3.64; CI: −15.41–44.22) vs serum starved (23.61 ± 5.08; CI: 1.77–45.45) organ cultures illustrates a higher proportion of acidified germ cells after serum removal (Fig. 7G). These findings indicate that maintaining newborn ovaries in the presence of serum lessens the incidence of autophagy. We next tested the effects of apoptosis or autophagy inhibitors on the density of LTR positive germ cells.

Autophagy as an effector of germ cell loss in the newborn ovary

3-Methyl adenine (3MA), a commonly used autophagy inhibitor (Seglen & Gordon 1982, Boya *et al.* 2005), and ZVAD, a commonly used pan-caspase inhibitor (Kim *et al.* 2001, Boya *et al.* 2005), were used to selectively impair autophagy or apoptosis in the organ culture system described above. Ovaries ($n=3$) were exposed to either inhibitor alone or a combination of both inhibitors for 24 h and processed for LTR staining. In the presence of 3MA, an apparent reduction in the density of acidified cells was evident. In contrast, ZVAD treatment had little discernible effect on the density of acidified cells compared to control ovaries (Fig. 7E and C respectively and Supplementary Figure 2). Interestingly, exposure to both 3MA and ZVAD (Fig. 7F) for 24 h reduced LTR-positive cell density to levels comparable with those observed in the 3MA treatment group (Fig. 7D and Supplementary Figure 2). These trends were confirmed by quantitative

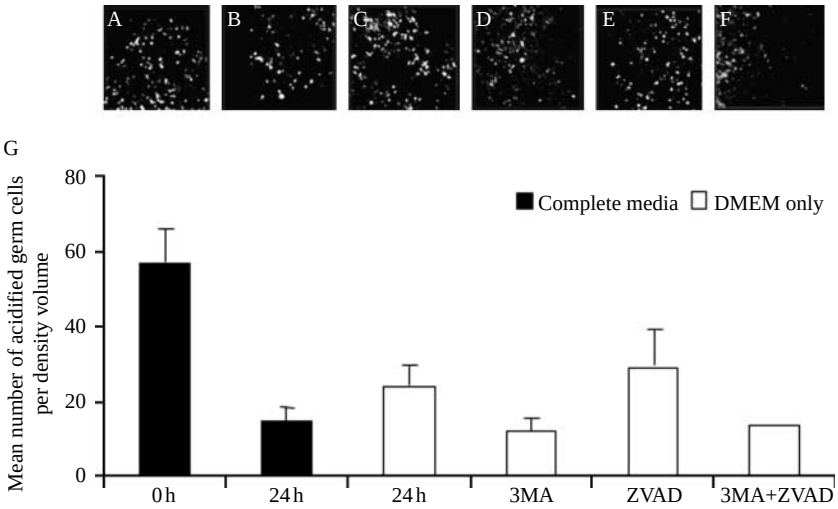

Figure 7 Quantitation of acidified germ cell density volume (G) in perinatal mouse ovaries cultured with (filled bars) and without (open bars) serum in the presence or absence of autophagy (3MA) and/or apoptosis (ZVAD) inhibitors (open bars). Panels at the top represent complete confocal projections of intact ovaries after image thresholding to reveal germ cell density for each treatment group. Note decreased prevalence of LTR positive cells when ovaries are maintained in complete medium for 0 h (A) and 24 h (B) compared to serum deprivation for 24 h in culture (C). The autophagy inhibitor 3MA, either alone (D) or in combination with ZVAD (F) reduces the density of acidified germ cells under serum free conditions. Note that treatment with ZVAD alone (E) has little effect on germ cell density compared to controls (C). (G) Values presented as mean ± S.E.M. ($n=3$ ovaries except for combination of the 2 inhibitors, for which $n=2$ ovaries).

image analysis (Fig. 7G). Whereas the density of LTR-positive cells was similar in control (24 h, 23.61 ± 5.08; CI: 1.77–45.45) and ZVAD (28.77 ± 8.86; CI: −9.36–66.90) groups, 3MA alone (12.05 ± 3.45; CI: −2.79–26.90) or in combination with ZVAD (13.38 ± 1.28; CI: −2.89–29.85) resulted in cell densities comparable with those attained after culture in the presence of serum (14.41 ± 3.64; CI: −15.41–44.22). Collectively, these results indicate that inhibition of autophagy and not apoptosis influence germ cell loss in newborn ovaries. This was further supported by an analysis of protein expression in perinatal ovaries (Fig. 8). Mediators of apoptosis (caspase 3, cathepsin D) and autophagy (LC-3B and beclin 1) were probed by western blots of pooled ovary samples from animals of E19.5, P2, P6 and P20 ages. Figure 8 shows active caspase 3 protein expression only on E19.5 (~17 kDa), and inactive caspase 3 expression (32 kDa) through all ages examined. Expression of the 16 kDa LC-3B, the autophagosomal membrane form of LC3B (Tanida *et al.* 2005), was evident between E19.5 and P6. The 36 kDa form of LC3B decreases at birth and remains relatively stable in expression at the later time points. Interestingly, cathepsin D gradually decreases until P20. Beclin levels show a slight decline at birth but also remain stable through to P20. Collectively, these data indicate that mediators of apoptosis and autophagy are differentially expressed while pre- and postnatal germ cell loss takes place.

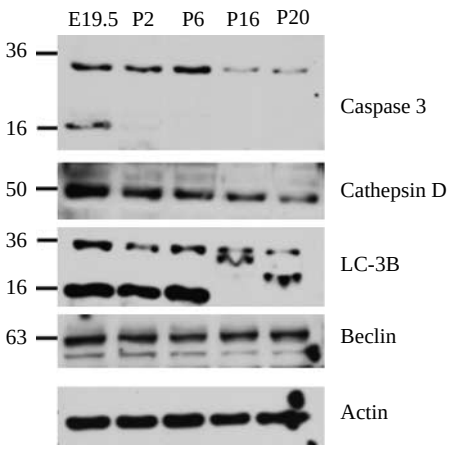

Figure 8 Apoptotic and autophagic protein expression. Western blot analysis of caspase 3 and cathepsin D (apoptosis), LC-3B and beclin 1 (autophagy), and β-actin expression in E19.5, P2, P6 and P20 pooled ovaries.

Discussion

Most female eutherian mammals establish a stockpile of follicle-enclosed oocytes at or soon after birth that is gradually depleted through the organisms' reproductive lifespan. In such species, it has long been appreciated that the most profound period of germ cell loss occurs shortly after parturition (Faddy & Gosden 2007). Moreover, it is generally held that the primary determinant of germ cell survival involves parallel processes of germ cell enclosure in the primordial follicle and selective susceptibility of some oocytes to type I PCD, apoptosis (Coucouvanis *et al.* 1993, De Pol *et al.* 1997, Reynaud & Driancourt 2000, Pepling & Spradling 2001, Tilly 2001). Since oocyte attrition is a fundamental tenet of reproductive aging and certain fertility disorders in women, there has been general interest in the mechanisms responsible for both the establishment and maintenance of the female germ cell supply (Gosden 2002, Matzuk 2005, Skinner 2005). Here, we used the commonly studied C57Bl/6 mouse strain to gain insight into the mechanisms of perinatal germ cell loss. While our results concur with previous studies indicating

that substantial germ cell attrition occurs at the onset of reproductive maturity and birth, they also question type I PCD as the sole cause for early germ cell loss. Instead, multiple perinatal mechanisms (apoptosis, germ cell extrusion, autophagy) interact to establish a finite follicle reserve for later use. The conclusion that different PCD mechanisms operate at discrete stages of ovarian development suggests a level of complexity in the regulation of ovarian germ cell and follicle survival that has only recently been appreciated (Lobascio *et al*. 2007).

Ovarian developmental transitions engage distinct PCD mechanisms

Unlike earlier histomorphometric analysis of germ cells and follicles in the mouse ovary, our methods using immunostaining of MVH for unequivocal germ cell identification provided an accurate counting method for analysis of all follicle stages from E15.5 to P150 (Fig. 2). This kind of analysis confirmed the sharp decline in oocyte number at birth noted by others (Baker 1966, Coucouvanis *et al*. 1993, Ratts *et al*. 1995, McClellan *et al*. 2003, Kerr *et al*. 2006) and further showed that over the postnatal period approaching puberty (P6–P42), little change in follicle numbers was detected. Our results are in agreement with those of Kerr *et al*. (2006) (Supplementary Table 1). Many studies have demonstrated the occurrence of germ cell loss through apoptosis in fetal mouse ovary (Coucouvanis *et al*. 1993, Pepling & Spradling 2001, De Felici *et al*. 2005) and in some cases have linked type I PCD to errors in meiotic cell cycle checkpoint control (Burgoyne & Baker 1985). It should be emphasized, however, that the frequency of TUNEL-positive oocytes seen in fetal mouse ovary is surprisingly small ranging from 0.5 to 5% (Pesce *et al*. 1997, Pepling & Spradling 2001). A recent re-examination of this stage of ovarian development by Lobascio *et al*. (2007) has concluded that previous studies failed to recognize the complexity of germ cell loss at fetal stages. Interestingly, these authors called attention to the fact that the mTOR inhibitor, rapamycin, increased TUNEL staining in germ cells presumably due to the negative effect of mTOR on autophagy. Moreover, calpain inhibitor 1 also induced apoptosis in germ cells after prolonged culture as evidenced by the appearance of caspase-negative and atypical TUNEL-positive oocytes (De Felici *et al*. 2007, Lobascio *et al*. 2007). The rare occurrence of apoptotic cells in perinatal ovaries described here further demonstrates that additional, PCD mechanisms, such as autophagy, are involved with germ cell attrition prior to and after birth in the mouse. These findings are immediately relevant to strategies seeking to prevent germ cell loss due to therapeutic or genetic causes and emphasize the need to clearly identify the PCD mechanisms involved.

Germ cell extrusion during ovarian morphogenesis

Oocytes have been noted to reside close to or within the ovarian epithelium in previous studies (Wordinger *et al*. 1990, Hirshfield 1992, Motta *et al*. 2003, Albertini & Barrett 2004) but the relative rarity of these and their disappearance with further postnatal development of the ovary have led most to conclude that this is a minor cause of germ cell loss (Byskov 1982, Hirshfield 1992, Motta *et al*. 2003). Estimates based on the present studies would tend to support this idea based upon analysis of static images at various time points. While this process could result in loss of a significant fraction of oocytes resident at the time of birth, a definitive assessment awaits development of technologies suitable for an analysis of this dynamic process.

Autophagy: an alternative mechanism for perinatal germ cell loss

The perinatal period of life in placental mammals marks an abrupt transition in energy homeostasis. Mounting evidence shows that up regulation of autophagy follows in the immediate hours of postnatal life in many tissues and organs (Kuma *et al*. 2004). It is now accepted that this burst of autophagy is an adaptive response to nutritional stress once the newborn organism is deprived of placental nutrients (Kuma *et al*. 2004). Coupled with studies on fetal ovaries cited above (De Felici *et al*. 2007, Lobascio *et al*. 2007), our results implicate autophagy in the establishment of the follicle reserve. That multiple PCD mechanisms would be involved in this process is not surprising since it has been shown in many other systems (Kuma *et al*. 2004). Moreover, ultrastructural studies of mammalian oocytes have repeatedly documented the presence of lysosomes and autophagosomes in many species (Hertig & Adams 1967, Wassarman & Albertini 1994) but the developmental time course of their appearance and its relationship to autophagy have not been established.

Lysosome amplification is a hallmark of autophagy (Bursch 2001). Using LAMP1 immunocytochemistry and vital markers for lysosomes, we document amplification of lysosomes in oocytes upon birth (Fig. 5). Moreover, accumulation of lysosomes was most apparent in oocytes enclosed within primordial follicles whereas these structures were less apparent in oocytes that were either extruded through the ovarian surface or undergoing PCD.

However, discriminating between germ cells actively undergoing autophagy or apoptosis required application of a vital staining method that would permit assessment of germ cell behavior at critical developmental stages. For this, we used the LTR method and confocal microscopy so that intact living ovaries could be evaluated by digital image analysis (Fig. 6). Fetal and neonatal ovaries exhibited cytoplasmic acidification of germ cells that were characterized by their size and disposition. These

germ cells were distinct from resident macrophages and smaller germ cells that exhibited fibrillar staining after LTR labeling. Quantitation of germ cells at (P0) and just after birth (P1) indicated a gradual decline in their density at this transition. Importantly, ovaries from newborn animals cultured in the presence of serum-enriched medium displayed fewer LTR positive germ cells than those maintained in a serum-free environment This finding suggests that the removal of ovary from a growth factor-rich environment, rather than an endogenous signal, was able to elicit germ cell death at the time when major loss is occurring. Growth factors are known to prevent follicle loss in both *in vivo* and *in vitro* models of ovarian development (De Felici 2000, Nilsson & Skinner 2001, Skinner 2005, Bristol-Gould *et al.* 2006) and one likely candidate for maintaining germ cells is Kit ligand (Parrott & Skinner 1999, De Felici 2000, Hutt *et al.* 2006). While this experimental approach would be helpful in defining the physiological factors that regulate follicle formation and survival under dynamic conditions, some studies involving section analysis have supported the general notion that growth factor deprivation at birth is responsible for much of the germ loss seen at this time (Durlinger *et al.* 1999, Bristol-Gould *et al.* 2006). Whether the effects of serum on germ cell loss were mediated by apoptosis or autophagy was next investigated using selective inhibitors for each of these processes.

As shown above (Fig. 7 and Supplementary Figure 2), the selective autophagy inhibitor 3MA (Seglen & Gordon 1982, Boya *et al.* 2005) reduced the acidified germ cell density compared to controls whereas no effect was seen after treatment with ZVAD, a pan-caspase inhibitor. Moreover, use of both inhibitors reduced the density of acidified germ cells to the same extent as 3MA alone, suggesting further that autophagy rather than apoptosis is involved in germ cell loss after birth. That combinatorial mechanisms may mediate this process is further indicated by western blot analysis of components for either apoptosis or autophagy (Fig. 8).

Prominent perinatal expression of beclin 1 and LC-3B (16 kDa) in ovaries at E19.5, P2 and P6 directly implicate autophagy, but, as also shown, activated caspase 3 (~17 kDa) is present only in fetal ovaries. Since cathepsin D is also present over this time interval, it is tempting to suggest that apoptosis at low levels may occur both prior to and following birth whereas the activation of autophagy appears to be directly linked to parturition-induced starvation. Caspase 3 antibody also detects a 36 kDa component that most likely is the inactive, constitutively expressed form that requires cleavage for protease activation (Boone & Tsang 1998). Moreover, 16 kDa band corresponding to the LC3B II isoform is localized to autophagosomes and autolysosomes (Tanida *et al.* 2005). Together, these results support the existence of combinatorial mechanisms for germ cell loss and survival at birth. Further resolution of the interactions between both of these PCD pathways will be facilitated by the use of conditional knockouts of genes involved in each of these processes.

In summary, these results suggest that multiple PCD mechanisms function in the perinatal mouse ovary to establish the primordial follicle reserve. The relative contributions of apoptosis and autophagy may vary at distinct stages of ovarian development. The present findings will be of relevance to emergent technologies seeking to optimize and preserve high quality oocytes for experimental or clinical use. Furthermore, they uncover a level of complexity in female germ line development in eutherian mammals not previously appreciated. Future studies seeking to rescue female germ cells in newborns, adolescents or adults will need to take into account the diverse mechanisms that are at play during different stages in the lifespan of the mammalian ovary.

Materials and Methods

Animals

Inbred C57Bl/6 mice (Charles River, or Taconic Farms Inc., Germantown, NY, USA) used for these experiments were housed in a 14 h light: 10 h dark environment at constant temperature. Food and water were provided *ad libitum*. All animals were killed by cervical dislocation. Mice were maintained and used in accordance with the policies of the University of Kansas Animal Care and Use Committee (protocol # 2007-1681).

Ovary collection and tissue preparation

Right and left ovaries from each animal were collected at embryonic (E15.5 and E19.5), prepubertal (P2, 6, 10, 12, 16, 20 days of age) and adult (P42, 100 and 150 days of age) stages. Five ovaries were collected at each stage from at least two separate litters. Intact bursa enclosed ovaries were fixed in either Bouin's fluid (Sigma; follicle counts) or 2% paraformaldehyde (PFA, Sigma; immunohistochemistry), for 4–6 h at room temperature and overnight at 4 °C respectively. Following fixation, ovaries were transferred to 70% ethanol and processed for paraffin embedding by standard techniques. For whole mount analysis, intact ovaries were fixed overnight in 2% formaldehyde microtubule stabilization buffer (MTSB-XF), (Messinger & Albertini 1991) at 4 °C and stored at 4 °C in a blocking buffer as previously described until use.

Immunocytochemistry

For follicle counts 5 μm sections were dewaxed and re-hydrated using conventional methods. Endogenous peroxidases were quenched in 0.3% hydrogen peroxide in methanol (5 min, room temperature). After three washes in Automation Buffer (Biomeda-Fisher, Foster City, CA, USA), slides were blocked for 30 min at room temperature in 9% goat serum (Zymed) containing 3% BSA (Sigma). Sections were labeled (overnight, 4 °C) with polyclonal rabbit

antibody against MVH, (a cytoplasmic germ cell specific protein, gift from Dr Noce) diluted 1:1200 in 1% BSA. Washed sections were then incubated with biotinylated goat anti-rabbit serum (Zymed; 1:200 in 1% BSA) for 30 min at room temperature, rinsed and incubated with HRP Avidin D (HRP; Vector, Burligame, CA, USA; 1:500) for 10 min. Diaminobenzidine was used as a substrate for HRP. Sections were counterstained with PAS reagent and Harris hematoxylin (Protocol) containing 4% acetic acid. Tissues were dehydrated, cleared and coverslipped using Permount (Fisher, Pittsburgh, PA, USA). Control slides were processed identically but primary antibody was omitted.

Follicle morphology and classification

Follicles were classified according to Pedersen & Peters (1968) and Myers *et al.* (2004). Briefly, follicles were classified as primordial if the oocytes were partially or completely surrounded by squamous GCs, equivalent to Type 1–2 in the Pedersen & Peters (1968) classification. Primary follicles were those exhibiting one complete layer of cuboidal GCs, (Type 3–3b). In the transition from primordial to primary classification was made according to the predominant type of GCs present. Secondary follicles were classified as all follicles having more than one GC layer and no visible antrum (Type 4–5). Follicles with a small antrum were designated early antral (Type 6) and antral follicles (Type 7) when the follicle had a single large antral space.

Follicle counting

Counts were made using a 40× Nikon objective in an Alphaphot 2 SY2 Nikon microscope. Follicles with an intact oocyte nucleus containing a dark nucleolus were counted in every second section and repeat scoring was unlikely because ~12 μm germ cells in primordial and primary follicles would not be present in the third 8 μm section used for sampling frequency in this study. For these follicle classes, final totals were derived by doubling the follicles recorded in every second section. However, for secondary to pre-ovulatory follicles this adjustment was omitted in order to limit over-representation (Liu *et al.* 2002). The position of primordial follicles relative to the ovarian surface epithelium was also determined and these were scored as extra-ovarian (if in the bursal cavity) or intraepithelial (if associated with or subjacent to the epithelium).

Static and dynamic imaging of germ cell loss

PCD was studied using a combination of methods to discriminate between apoptosis and autophagy in histological samples or ovarian whole mounts.

Apoptosis detection

TUNEL was used to detect apoptosis following the manufacturer's protocol for the ApopTag Peroxidase *In Situ* apoptosis detection kit (Chemicon-Millipore, Billerica, MA, USA). For active caspase 3 (1:100; R&D Systems, cat. # AF835), and poly (ADP ribose) polymerase (cleaved-PARP form; 1:50; AbCam cat. # ab32064) paraffin sections of PFA fixed ovaries were dewaxed and microwaved (high, 15 min) in sodium citrate buffer (0.01 M, pH 6.0) for antigen retrieval. After sections were washed and blocked they were incubated in primary antibody (see above). For fluorescent imaging, slides were exposed to Alexa-fluor goat anti-rabbit secondary antibodies (488 or 568; 1:800; Molecular Probes, Invitrogen; 37 °C; 1 h) rinsed (3×), incubated in Hoechst 33258 (1 μg/ml; Polysciences Inc., Warrington, PA, USA) for nuclear staining and mounted in Prolong Anti-fade Reagent (Molecular Probes, Invitrogen). Control slides were prepared as above by omitting primary antibody.

Whole mount preparations of intact or fragments of ovaries fixed in MTSB-XF (Messinger & Albertini 1991) were processed for confocal microscopy using the same primary and secondary antibodies noted above. These were diluted with wash solution and used with constant agitation for 24 h at 4 °C. Multiple wash steps (a total of 4 h, several changes; 37 °C) separated primary and secondary antibody incubations. Hoechst 33258 (nuclei) and Alexa 546 Phalloidin (f-actin; 1:100 dilution, Molecular Probes, Invitrogen) were used to define nuclear and cell boundaries. Ovaries were mounted on microscope slides in glycerol/PBS containing Hoechst 33258.

LTR is an aldehyde-fixable dye that concentrates in acidic membrane-bound intracellular compartments of living tissue, which has been adopted by Zucker *et al.* (1998) as an assay for PCD in rodent tissues. We modified the protocol as follows: intact ovaries were incubated in 5 μM LTR (Molecular Probes, Invitrogen) in MEM (Gibco, Invitrogen) for 1 to 2 h at 37 °C and 5% CO_2. After two washes in PBS the ovaries were fixed in a mixture of 4% PFA and 1% glutaraldehyde (Sigma) in PBS (2 h; 37 °C with shaking, followed by 4 °C, overnight), rinsed twice in PBS and dehydrated in a series of methanol/PBS solutions (50, 70, 95 and 100%-2×; 15 min each). Tissues were then cleared in a 1:2 mixture of benzyl alcohol to benzyl benzoate (BABB, Sigma). From absolute methanol, tissues were exchanged through 50% and 70% BABB-methanol and finally pure BABB solution (2 h each; at room temperature with constant agitation). For confocal microscopy, a single ovary was centrally positioned in a metal washer sealed by Permount (Fisher) to the center of a glass bottomed tissue culture dish (Delta T, Fisher). The depression was filled with BABB and a coverslip applied with Permount (Fisher).

Lysosome detection and autophagy

Both immunohistochemistry and live cell imaging were used to visualize the lysosomes and autophagic markers. Lysosome abundance and distribution in developing germ cells was determined using a rat antibody to LAMP1 (a lysosomal membrane glycoprotein; Developmental Studies Hybridoma Bank, University of Iowa, 1:100, cat. # 1D4B), and goat anti-rat Alexa Fluor 488 secondary antibody in both paraffin sections and whole mounts as described above. For analysis of acidic organelles in living tissues, intact ovaries (embryonic 19.5 and post–natal days 0 (day of birth), 1 and 2) were cultured for 4 and 18 h in Acridine Orange (1 μg/ml;

Molecular Probes, Invitrogen) or LysoSensor Yellow/Blue DND (10 μg/ml; Molecular Probes, Invitrogen). Individual ovaries were cultured in 100 μl drops of culture media (MEM with Earle's Salts and L-glutamine without phenol red (Gibco); 10% FCS (Hyclone, Logan, UT, USA), 100 ng/ml of leukemia inhibitory factor (Sigma), 100 ng/ml of stem cell factor (SCF/c-Kit, PreProtech), 50 ng/ml of IGF1 (Sigma), and 1% gentomycin (Sigma)), covered with mineral oil (Sigma) and incubated with at 37 °C with 5% CO_2. Glass bottom culture dishes allow live imaging of intact ovaries after 4 or 18 h of culture and Hoechst 33342 was used as a vital nuclear stain. LAMP1 was quantified using Integrated Morphometry Analysis (IMA) software (MetaMorph version 7.5 Universal Image Corporation, USA), to define individual germ cell boundaries (regions of interest) and measuring the area occupied by the LAMP1 signal. Values are represented as the percentage of LAMP1 per germ cell.

In vitro *starvation of neonatal ovaries*

To create *in vitro* conditions resembling the nutrient-deprived state of neonatal ovaries, P0 and P1 ovaries (3 per group from different litters) were incubated in 600 μl complete organ culture media (DMEM:Ham's F12, 1:1; 10% FBS, 1% ITS (Sigma), 1% penicillin/streptomycin (P/S, Sigma)), suspended in a 3 μm PCF insert Millicell (Millipore), and placed in a 24 well tissue culture plate. Following a stabilization period, ovaries were either incubated for an additional 21 h in complete media or starvation media (DMEM+1% P/S). At the end of the total 24 h incubation period, LTR (1%) was added to each well. For 0 h control ovaries, LTR was added for 3 h following the initial stabilization period. Hoechst 33342 was typically added as a nuclear counterstain. Ovaries were washed in PBS and fixed as for LTR in 4% PFA prior to confocal microscopy without BABB clearing.

Effect of apoptosis and autophagy inhibitors on starved neonatal ovaries

Using complete or starvation media (as above), E19.5 or P0 ovaries were incubated with inhibitors of PCD. For apoptosis the pan-caspase Inhibitor 1 ZVAD (OME)-FMK (50 μM; Calbiochem-EMD, Gibbstown, NJ, USA) was used; for autophagy, 3MA (10 mM; Sigma) was used. Drugs were added alone or in combination for 21 h and LTR and Hoechst 33342 were added for the final 3 h of incubation. Tissues were analyzed by confocal microscopy to determine the volume density of LTR labeled germ cells in Z-section composites of whole ovaries using IMA software as above. Briefly, LTR positive objects with a diameter greater than 80 μm^2 (determined to be the approximate diameter of germ cells) were thresholded and counted within 3-dimensional projections of a single ovary from each treatment group ($n=3$ per group). Approximately, 50% of the entire ovary volume was assayed for each replicate in each treatment group and the data are expressed as mean density volume per group.

Western blot analysis of apoptosis and autophagy proteins

Ovaries from fetal (E19.5), neonatal (P0, P1, P2, and P6), and pubertal (P20) mice were washed in cold PBS (4 °C), and lysed (20 mM Tris–HCl (pH 7.4; Sigma), 150 mM of sodium chloride (Sigma), 1 mM of EDTA (Sigma), 1 mM EGTA (Sigma), 1% Triton, 2.5 mM sodium pyrophosphate (Sigma), 1 mM β-glycerolphosphate, 1 mM sodium orthovanadate (Sigma), 1 μg/ml leupetin (Sigma), 1 mM phenylmethanesulfonyl fluoride (Sigma)). Twenty micrograms of protein from pooled samples (E19.5, P2, 6, 16, and 20) was loaded on 12% SDS–Page gels and transferred to a PVDF membrane (Millipore). Membranes were incubated for 1 h in PBS-Tween 20 (0.05%) containing 5% nonfat milk and probed with primary antibodies to caspase 3 (Cell Signaling cat. # 9661), LC-3B (Cell Signaling cat. # 2775), cathepsin D (Santa Cruz, cat. # 10725) and beclin 1 (Sigma cat. # B6061) followed by incubation with the appropriate HRP-conjugated secondary antibody (Southern Biotechnologies Associates, Birmingham, AL, USA); bands were detected with SuperSignal West Pico chemiluminescent substrate (Pierce, Rockford, IL, USA) and an anti-actin antibody (Sigma cat. # A2066) was used to verify comparable loading between samples.

Image acquisition and analysis

Whole mount or sectioned ovary preparations were imaged using a LSM-510 Pascal confocal microscope (Zeiss) mounted on a Zeiss Axiovert 200M microscope equipped with excitation specific Diode (405 nm), Argon (458, 477, 488 and 514 nm) and Helium Neon lasers (594 nm). Single scans or Z series data sets were made using 20×, 40× and 63× (NA=1.25) objectives and data were archived and analyzed using LSM software. Bright field images were obtained using a Nikon Eclipse 8i microscope with 20× and 100× objectives and a Sony XWaveHAD color camera.

Statistical analysis

Data are presented as mean±S.E.M. and statistical analysis of follicle number counts was performed using Prism 4.0 (GraphPad Software Inc., San Diego, CA, USA). Data were analyzed by calculation of CI and non-parametric Kruskal–Wallis test of significance. Data were considered statistically significant in cases where $P<0.05$.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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